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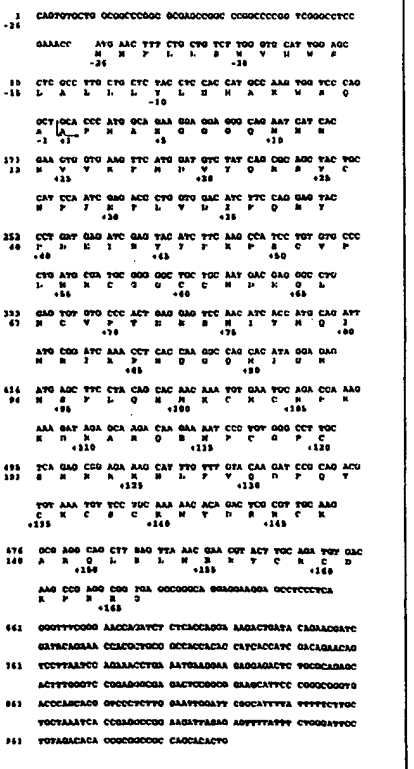
(57) Abstract

The present invention involves the preparation of vascular endothelial growth factor (VEGF) antagonist molecules comprising variant VEGF polypeptides which are capable of binding to and occuyping cell surface VEGF receptors without inducing a VEGF response, thereby antagonizing the biological activity of the native VEGF protein. Specifically, the variant VEGF polypeptides of the present invention comprise modifications of at least one cysteine residue in the native VEGF sequence, thereby inhibiting the ability of the variant polypeptide to dimerize through the formation of disulfide bonds. The present invention is further directed to methods for preparing such variant VEGF antagonists and to methods, compositions and assays utilizing such variants for producing pharmaceutically active materials having therapeutic and pharmacologic properties that differ from the native VEGF protein.



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VARIANTS OF VASCULAR ENDOTHELIAL CELL GROWTH FACTOR HAVING ANTAGONISTIC PROPERTIES

FIELD OF THE INVENTION

The present invention is directed to particular variants of vascular endothelial cell growth factor (hereinafter sometimes referred to as VEGF) which bind to and occupy cell surface VEGF receptors without inducing a VEGF response, thereby antagonizing the biological activity of the native VEGF protein. The present invention is further directed to methods for preparing such variant VEGF antagonists and to methods, compositions and assays utilizing such variants for producing pharmaceutically active materials having therapeutic and pharmacologic properties that differ from the native VEGF protein.

BACKGROUND OF THE INVENTION

The two major cellular components of the mammalian vascular system are the endothelial and smooth muscle cells. Endothelial cells form the lining of the inner surface of all blood vessels in the mammal and constitute a non-thrombogenic interface between blood and tissue. Therefore, the proliferation of endothelial cells is an important component for the development of new capillaries and blood vessels which, in turn, is a necessary process for the growth and/or regeneration of mammalian tissues.

One protein that has been shown to play an extremely important role in promoting endothelial cell proliferation and angiogenesis is vascular endothelial cell growth factor (VEGF). VEGF is a heparin-binding endothelial cell-specific growth factor which was originally identified and purified from media conditioned by bovine pituitary follicular or folliculostellate (FS) cells. Ferrara and Henzel, *Biochem. Biophys. Res. Comm.* 161:851-858 (1989). Naturally-occurring VEGF is a dimeric protein having an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. Normal dimerization between individual native VEGF monomers occurs through the formation of disulfide bonds between the cysteine residues located at amino acid position 51 of one monomeric unit bonding to the cysteine residue at amino acid position 60 of another monomeric unit and vice versa. Human VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 165, 189 and 206 amino acids per monomer), wherein each form arises as a result of alternative splicing of a single RNA transcript. For example, $VEGF_{121}$ is a soluble mitogen that does not bind heparin whereas the longer forms of VEGF bind heparin with progressively higher affinity.

Biochemical analyses have shown that the native VEGF dimer exhibits a strong mitogenic specificity for vascular endothelial cells. For example, media conditioned by cells transfected by human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas medium conditioned by control cells did not. Leung et al., *Science* 246:1306 (1989). Thus, the native VEGF dimer is known to promote vascular endothelial cell proliferation and angiogenesis, a process which involves the formation of new blood vessels from preexisting endothelium. As such, the native VEGF may be useful for the therapeutic treatment of numerous conditions in which a growth-promoting activity on the vascular endothelial cells is important, for example, in ulcers, vascular injuries and myocardial infarction.

The endothelial cell proliferative activity of the VEGF dimer is known to be mediated by two high affinity tyrosine kinase receptors, flt-1 (FMS-like tyrosine kinase) and KDR (kinase domain region), which exist only on the surface of vascular endothelial cells. DeVries, et al., *Science* 225:989-991 (1992) and Terman, et al., *Oncogene* 6:1677-1683 (1991). As cells become depleted in oxygen, because of trauma and the like, VEGF production increases in such cells, wherein the generated VEGF protein subsequently binds to its respective cell surface receptors in order to signal ultimate biological effect. The signal then increases vascular permeability and the cells divide and expand to form new vascular pathways. Thus, native VEGF functions to induce vascular proliferation through the binding to endothelial cell-specific receptors.

While VEGF-induced vascular endothelial cell proliferation is desirable under certain circumstances, vascular endothelial cell proliferation and angiogenesis are also important components of a variety of diseases and disorders. Such diseases and disorders include tumor growth and metastasis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, hemangiomas, immune rejection of transplanted corneal tissue and other tissues, and chronic inflammation. Obviously, in individuals suffering from any of these disorders, one would want to have a means for inhibiting, or at least substantially reducing, the endothelial cell proliferating activity of the native VEGF dimeric protein.

Having an available means for inhibiting native VEGF activity is important for a number of reasons. For example, in the specific case of tumor cell growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia and for providing nourishment to the growing solid tumor. Folkman, et al., *Nature* 339:58 (1989). Angiogenesis also allows tumors to be in contact with the vascular bed of the host, which may provide a route for metastasis of tumor cells. Evidence for the role of angiogenesis in tumor metastasis is provided, for example, by studies

showing a correlation between the number and density of microvessels in histologic sections of invasive human breast carcinoma and actual presence of distant metastasis. Weidner et al., *New Engl. J. Med.* 324:1 (1991). Thus, one possible mechanism for the effective treatment of neoplastic tumors is to inhibit or substantially reduce the endothelial cell proliferative and angiogenic activity of the native dimeric VEGF protein.

Therefore, in view of the role that VEGF-induced vascular endothelial cell growth and angiogenesis play in many diseases and disorders, it is desirable to have a means for reducing or substantially inhibiting one or more of the biological effects of the native VEGF protein, for example, the mitogenic or angiogenic effect thereof. Thus, the present invention is predicated upon research intended to identify novel VEGF variant polypeptides which are capable of inhibiting one or more of the biological activities of native VEGF. Specifically, the present invention is predicated upon the identification of VEGF variants which are capable of binding to and occupying cell-surface VEGF receptors without inducing a typical VEGF response, thereby effectively reducing or substantially inhibiting the effects of native VEGF. It was postulated that if one could prepare such VEGF variants, one could use such variants in instances of tumor treatment in order to starve the tumors for intended regression.

It was a further object of this research to produce VEGF variants which lose the ability to properly dimerize through the formation of covalent cysteine-cysteine disulfide bonds. Such variants include variant VEGF monomers which lack the ability to dimerize through the formation of cysteine-cysteine disulfide bonds and variant VEGF monomers which may dimerize through the formation of at least one cysteine-cysteine disulfide bond, however, wherein at least one disulfide bond differs from that existing in the native VEGF dimer. Such variants possess the ability to bind to and occupy cell surface VEGF receptors without inducing a VEGF response, thereby competing with native VEGF for binding to the receptors and antagonistically inhibiting the biological activity of the native VEGF dimer.

As further objects, the VEGF variants of the present invention can be employed in assays systems to discover small molecule agonists and antagonists for intended therapeutic use.

The results of the above described research is the subject of the present invention. We herein demonstrate that mutation or modification of the cysteine residues at amino acid positions 51 and/or 60 of the native VEGF amino acid sequence functions to produce VEGF variants which lose the ability to properly dimerize. Specifically, substitution of cysteine at positions 51 and/or 60 with another amino acid or modification of the cysteine at that site prevents the ability of that amino acid to participate in the formation of a disulfide bond. These variants, however, retain the ability to bind to and occupy cell surface VEGF receptors without inducing a VEGF response, thereby effectively inhibiting the biological activity of the native VEGF dimer.

SUMMARY OF THE INVENTION

The present invention provides variants of the native VEGF protein which are capable of binding to a VEGF receptor on the surface of vascular endothelial cells, thereby occupying those binding sites and inhibiting the mitogenic, angiogenic or other biological activities of the native VEGF protein. The novel antagonist molecules of the present invention, therefore, are useful for the treatment of diseases or disorders characterized by undesirable excessive vascularization, including by way of example, tumors, and especially solid malignant tumors, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation. The antagonists of the present invention are also useful for the treatment of diseases or disorders characterized by undesirable vascular permeability, such as edema associated with brain tumors, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial effusion (such as that associated with pericarditis) and pleural effusion.

In a preferred embodiment, the variant VEGF polypeptides of the antagonist molecules of the present invention comprise amino acid modifications of at least one cysteine residue present in the native VEGF amino acid sequence wherein modification of that cysteine residue(s) results in the polypeptide being incapable of properly dimerizing with another VEGF polypeptide.

In a particularly preferred embodiment, the cysteine residues of the native VEGF amino acid sequence that are modified are at amino acid positions 51 and/or 60 of the native VEGF amino acid sequence.

The novel VEGF variant polypeptides of the present invention may be recombinantly generated by creating at least one amino acid mutation at a cysteine residue in the native VEGF amino acid sequence such that the variant is incapable of properly dimerizing. Typical mutations include, for example, substitutions, insertions and/or deletions. The cysteine residue(s) of interest may also be chemically modified so as to be incapable of participating in a disulfide bond.

In other embodiments, the present invention is directed to isolated nucleic acid sequences encoding the novel VEGF antagonist molecules of the present invention and replicable expression vectors comprising those nucleic acid sequences.

In still other embodiments, the present invention is directed to host cells which are transfected with the replicable expression vectors of the present invention and are capable of expressing those vectors.

In yet another embodiment, the present invention is directed to a composition for treating indications wherein anti-angiogenesis is desired, such as in arresting tumor growth, comprising a therapeutically effective amount of the antagonist molecule of the present invention compounded with a pharmaceutically acceptable carrier. Another embodiment of the present invention is directed to a method of treating comprising

administering a therapeutically effective amount of the above described composition.

Expanding on the basic premise hereof of the discovery and mutagenesis of the native VEGF polypeptide to produce variant VEGF polypeptides, the present invention is directed to all associated embodiments deriving therefrom, including recombinant DNA materials and processes for preparing such variants, materials and information for compounding such variants into pharmaceutically finished form and assays using such variants to screen for candidates that have agonistic or antagonistic properties with respect to the native VEGF polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict both the amino acid and DNA sequence for a native VEGF protein having 165 amino acids. Predicted amino acids of the protein are shown below the DNA sequence and are numbered from the first residue of the N-terminus of the protein sequence. Negative amino acid numbers refer to the presumed leader signal sequence or pre-protein, while positive numbers refer to the putative mature protein.

Figure 2 is a schematic diagram showing the native VEGF dimer molecule having disulfide bonds between cysteine residues at amino acid positions 51 and 60 and 60 and 51, respectively, of the monomeric units, variant polypeptide C51D, wherein the cysteine residue at amino acid position 51 has been substituted by an aspartic acid residue resulting in the formation of a staggered dimer, variant polypeptide C60D, wherein the cysteine residue at amino acid position 60 has been substituted by an aspartic acid residue resulting in the formation of a staggered dimer and variant polypeptide C51D, C60D, wherein the cysteine residues at both amino acid positions 51 and 60 have been substituted by aspartic acid residues, thereby preventing disulfide bond formation and dimerization.

Figure 3 is a graph showing the binding profiles of native VEGF dimer ("•"), the staggered dimer formed from the C60D variant VEGF polypeptide ("□"), the staggered dimer formed from the C51D variant VEGF polypeptide ("○") and the monomeric VEGF variant polypeptide C51D, C60D ("Δ") to the KDR receptor. Data is presented as the ratio of bound polypeptide to free versus the picomolar (pM) concentration of unlabeled competitor.

Figure 4 is a graph showing the binding profiles of native VEGF dimer ("•") and the monomeric VEGF variant polypeptide C51D, C60D ("▲") to the KDR receptor. Data is presented as the ratio of bound polypeptide to free versus the nanomolar (nM) concentration of unlabeled VEGF competitor.

Figure 5 is a graph showing the binding profiles of native VEGF dimer ("•"), the staggered dimer formed from the C60D variant VEGF polypeptide ("■"), the staggered dimer formed from the C51D variant VEGF polypeptide ("○") and the monomeric VEGF variant polypeptide C51D, C60D ("▲") to the FLT-1 receptor. Data is presented as the ratio of bound polypeptide to free versus the nanomolar (nM) concentration of unlabeled VEGF competitor.

Figure 6 is a graph showing the binding profiles of native VEGF dimer ("•") and the monomeric VEGF variant polypeptide C51D, C60D ("■") to the FLT-1 receptor. Data is presented as the ratio of bound polypeptide to free versus the nanomolar (nM) concentration of unlabeled VEGF competitor.

Figure 7 is a graph demonstrating the ability of the native VEGF dimer ("•"), the staggered dimer formed from the C60D variant VEGF polypeptide ("○"), the staggered dimer formed from the C51D variant VEGF polypeptide ("Δ") and the monomeric VEGF variant polypeptide C51D, C60D ("□") to stimulate mitogenesis in endothelial cells. Data is presented as the total number of endothelial cells versus the picomolar (pM) concentration of polypeptide employed.

Figure 8 is a graph demonstrating the ability of the anti-VEGF monoclonal antibody A461 ("■") and the monomeric VEGF variant polypeptide C51D, C60D ("•") to inhibit VEGF-induced growth of endothelial cells. Data is presented as the total number of endothelial cells versus the ratio of antibody or monomer inhibitor to VEGF employed.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "vascular endothelial cell growth factor," or "VEGF," refers to a native mammalian growth factor as defined in U.S. Patent 5,332,671, including the human amino acid sequence shown in Figure 1 and naturally occurring allelic and processed forms of such growth factors. VEGF proteins can exist in either monomeric or multimeric (for example, dimeric) form. "Proper dimerization" is the dimerization which normally occurs between native VEGF monomers.

The term "native" with regard to a VEGF protein refers to a naturally occurring VEGF protein of any human or non-human animal species, with or without the initiating methionine, whether purified from the native source, synthesized, produced by recombinant DNA technology or by any combination of these and/or other methods. Native VEGF proteins naturally exist as dimeric molecules, wherein the monomeric units thereof are covalently connected through the formation of cysteine-cysteine disulfide bonds. Native VEGF specifically includes the native human VEGF protein having the amino acid sequence shown in Figure 1 and possesses the ability to induce the proliferation of vascular endothelial cells *in vivo*.

The term "variant" with respect to a VEGF protein refers to a VEGF protein that possesses at least one amino acid mutation or modification (i.e., alteration) as compared to a native VEGF protein and which may or may not lack one or more of the biological activities of a native VEGF protein. Variant VEGF proteins generated by "amino acid modifications" can be produced, for example, by substituting, deleting, inserting and/or chemically modifying at least one amino acid in the native VEGF amino

acid sequence. Methods for creating such VEGF variants are described below.

The term "monomeric variant", "monomeric antagonist" or grammatical equivalents thereof refers to a variant VEGF protein having at least one amino acid alteration as compared to a native VEGF monomer, wherein said amino acid alteration acts to prevent dimer formation between the monomeric units. Thus, the "monomeric variants" or "monomeric antagonists" of the present invention are those VEGF variants which are incapable of dimerizing through the formation of cysteine-cysteine disulfide bonds. Monomeric variants of the native VEGF protein, however, will possess the ability to bind to and occupy cell-surface VEGF receptors without inducing a mitogenic and/or angiogenic VEGF response, although the binding affinity of the monomeric variant at those receptors may differ from that of a native VEGF protein.

The term "staggered dimer", "staggered antagonist" or grammatical equivalents thereof refers to a variant VEGF protein having at least one amino acid alteration as compared to a native VEGF protein and which retains the ability to dimerize through the formation of at least one cysteine-cysteine disulfide bond, however, where at least one of the disulfide bonds formed is different from that which exists in the native VEGF dimeric protein.

A "functional derivative" of a polypeptide is a compound having a qualitative biological activity, or lack thereof, in common with the another polypeptide. Thus, for example, a functional derivative of a VEGF antagonist compound of the present invention is a compound that has a qualitative biological activity in common with an original polypeptide antagonist, for example, as being capable of binding to cell surface VEGF receptors without inducing a VEGF response, thereby occupying those receptors and inhibiting native VEGF activity. "Functional derivatives" include, but are not limited to, amino acid sequence variants of the variant

VEGF proteins of the present invention, fragments of polypeptides from any animal species (including humans), derivatives of human and non-human polypeptides and their fragments, and peptide analogs of native polypeptides, provided that they have a biological activity, or lack thereof, in common with a respective variant VEGF protein. "Fragments" comprise regions within the sequence of a mature polypeptide. The term "derivative" is used to define amino acid sequence variants, and covalent modifications of a polypeptide.

"Identity" or "homology" with respect to a polypeptide and/or its functional derivatives is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art.

The term "biological activity" in the context of the definition of functional derivatives is defined as the possession of at least one function qualitatively in common with another polypeptide. The functional derivatives of the polypeptide antagonists of the present invention are unified by their qualitative ability to bind to a VEGF receptor without inducing a VEGF response, thereby preventing native VEGF from binding at that site and, in turn, inhibiting the biological activity of the native VEGF protein.

The term "antagonist" is used to refer to a molecule inhibiting a biological activity of a native VEGF protein. Preferably, the VEGF antagonist compounds herein inhibit the ability of VEGF to induce vascular endothelial cell proliferation. Preferred antagonists essentially completely inhibit vascular endothelial cell proliferation.

Ordinarily, the terms "amino acid" and "amino acids" refer to all naturally occurring L-α-amino acids. In some embodiments, however, either D-amino acids or non-natural substituted amino acids may be present in the polypeptides or peptides of the present invention in order to facilitate conformational restriction. For example, in order to facilitate disulfide bond formation and stability, a D-amino acid cysteine may be provided at one or both termini of a peptide functional derivative or peptide antagonist of the native VEGF protein. The amino acids are identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan
Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

These amino acids may be classified according to the chemical composition and properties of their side chains. They are broadly classified into two groups, charged and uncharged. Each of these groups is divided into subgroups to classify the amino acids more accurately:

I. Charged Amino Acids

Acidic Residues: aspartic acid, glutamic acid

Basic Residues: lysine, arginine, histidine

II. Uncharged Amino Acids

Hydrophilic Residues: serine, threonine, asparagine, glutamine

Aliphatic Residues: glycine, alanine, valine, leucine, isoleucine

Non-polar Residues: cysteine, methionine, proline

Aromatic Residues: phenylalanine, tyrosine, tryptophan

The term "amino acid sequence variant" or "amino acid alteration" refers to molecules having at least one differences in their amino acid sequence as compared to another amino acid sequence, usually the native amino acid sequence.

"Substitutional" variants are those that have at least one amino acid residue in a corresponding sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

"Insertional" variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a corresponding sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid.

"Deletional" variants are those with one or more amino acids in a corresponding amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

The term "isolated" means that a nucleic acid or polypeptide is identified and separated from contaminant nucleic acids or polypeptides present in the animal or human source of the nucleic acid or polypeptide.

Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Yet another example is hybridization using a buffer of 10% dextran sulfate, 2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

"Transfection" refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N. *Proc. Natl. Acad. Sci. (USA), 69,* 2110 (1972) and Mandel *et al. J. Mol. Biol. 53,* 154 (1970), is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham, F. and

van der Eb, A., *Virology, 52*, 456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued August 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen, P., *et al. J. Bact., 130*, 946 (1977) and Hsiao, C.L., *et al. Proc. Natl. Acad. Sci. (USA) 76*, 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

"Site-directed mutagenesis" is a technique standard in the art, and is conducted using a synthetic oligonucleotide primer complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the single-stranded phage DNA, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage. Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. Plaques of interest are selected by hybridizing with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then selected, sequenced and cultured, and the DNA is recovered.

"Operably linked" refers to juxtaposition such that the normal function of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences and wherein the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. For example, DNA for a presequence or secretory leader is operably linked to DNA for a

polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. To effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

As used herein, "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 mg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 μl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis *et al.* 1982, *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982) pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see R. Lawn *et al., Nucleic Acids Res. 9,* 6103-6114 (1981), and D. Goeddel *et al., Nucleic Acids Res. 8,* 4057 (1980).

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (T. Maniatis *et al.* 1982, supra, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 mg of approximately equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis *et al.* 1982, supra, p. 90, may be used.

"Oligonucleotides" are short-length, single- or double- stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP Pat. Pub. No. 266,032 published May 4, 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al., Nucl. Acids Res. 14,* 5399-5407 [1986]). They are then purified on polyacrylamide gels.

The abbreviation "KDR" refers to the kinase domain region of the VEGF molecule, whether a native VEGF molecule or a variant thereof. It is this region which is known to bind to the kinase domain region receptor.

The abbreviation "FLT-1" refers to the FMS-like tyrosine kinase binding domain which is known to bind to the corresponding flt-1 receptor. These receptors exist on the surfaces of endothelial cells.

B. General Methodology

1. Glycosylation

The VEGF variants of the present invention may contain at least one amino acid sequence that has the potential to be glycosylated through an N-linkage and that is not normally glycosylated in the native VEGF molecule.

Introduction of an N-linked glycosylation site in the variant requires a tripeptidyl sequence of the formula: asparagine-X-serine or asparagine-X-threonine, wherein asparagine is the acceptor and X is any of the twenty genetically encoded amino acids except proline, which prevents glycosylation. See D.K. Struck and W.J. Lennarz, in *The Biochemistry of Glycoproteins and Proteoglycans*, ed. W.J. Lennarz, Plenum Press, 1980, p. 35; R.D. Marshall, *Biochem. Soc. Symp., 40*, 17 (1974), and Winzler, R.J., in *Hormonal Proteins and Peptides* (ed. Li, C.I.) p. 1-15 (Academic Press, New York, 1973). The amino acid sequence variant herein is modified by substituting for the amino acid(s) at the appropriate site(s) the appropriate amino acids to effect glycosylation.

If O-linked glycosylation is to be employed, O-glycosidic linkage occurs in animal cells between N-acetylgalactosamine, galactose, or xylose and one of several hydroxyamino acids, most commonly serine or threonine, but also in some cases a 5-hydroxyproline or 5-hydroxylysine residue placed in the appropriate region of the molecule.

Glycosylation patterns for proteins produced by mammals are described in detail in *The Plasma Proteins: Structure, Function and Genetic Control*, F.W. Putnam, ed., 2nd edition, volume 4 (Academic Press, New York, 1984), p. 271-315, the entire disclosure of which is incorporated herein

by reference. In this chapter, asparagine-linked oligosaccharides are discussed, including their subdivision into at least three groups referred to as complex, high mannose, and hybrid structures, as well as O-glucosidically linked oligosaccharides.

Chemical and/or enzymatic coupling of glycosides to proteins can be accomplished using a variety of activated groups, for example, as described by Aplin and Wriston in *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981), the disclosure of which is incorporated herein by reference. The advantages of the chemical coupling techniques are that they are relatively simple and do not need the complicated enzymatic machinery required for natural O- and N-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine or histidine, (b) free carboxyl groups such as those of glutamic acid or aspartic acid, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described more fully in PCT WO 87/05330 published September 11, 1987, the disclosure of which is incorporated herein by reference.

Glycosylation patterns for proteins produced by yeast are described in detail by Tanner and Lehle, *Biochim. Biophys. Acta, 906*(1), 81-99 (1987) and by Kukuruzinska *et al., Annu. Rev. Biochem., 56*, 915-944 (1987), the disclosures of which are incorporated herein by reference.

2. Amino Acid Sequence Variants

a. Additional Mutations

For purposes of shorthand designation of the VEGF variants described herein, it is noted that numbers refer to the amino acid residue/position along the amino acid sequences of the putative mature VEGF protein shown in Figures 1A and 1B.

The present invention is directed to variants of VEGF where such variants have modifications in the amino acid sequence that affect the ability of the VEGF monomeric units to properly dimerize. These variants have the ability to bind to and occupy cell-surface VEGF receptors without substantially activating vascular endothelial proliferation and angiogenesis, thereby inhibiting the biological activity of native VEGF. Specifically, amino acid modifications can be made at amino acid positions 51 and/or 60, each of which affect the ability of the variant VEGF monomers to properly dimerize. Moreover, additional variants based upon these original variants can be made by means generally known well in the art and without departing from the spirit of the present invention.

With regard to the VEGF variants of the present invention, for example, covalent modifications can be made to various of the amino acid residues.

b. DNA Mutations

Amino acid sequence variants of VEGF and variants thereof can also be prepared by mutations in the DNA. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown in Figure 1. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see EP 75,444A).

At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the VEGF, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed VEGF variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, site-specific mutagenesis.

Preparation of VEGF variants in accordance herewith is preferably achieved by site-specific mutagenesis of DNA that encodes an earlier prepared variant or a nonvariant version of the protein. Site-specific mutagenesis allows the production of VEGF variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by publications such as Adelman *et al., DNA 2,* 183 (1983), the disclosure of which is incorporated herein by reference.

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA*, Editor A. Walton, Elsevier, Amsterdam (1981), the disclosure of which is incorporated herein by reference. These phage are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-

stranded phage origin of replication (Veira *et al., Meth. Enzymol., 153,* 3 [1987]) may be employed to obtain single-stranded DNA.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea *et al., Proc. Natl. Acad. Sci. (USA), 75,* 5765 (1978). This primer is then annealed with the single-stranded protein-sequence-containing vector, and subjected to DNA-polymerizing enzymes such as E. coli polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as JM101 cells and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated protein region may be removed and placed in an appropriate vector for protein production, generally an expression vector of the type that may be employed for transformation of an appropriate host.

c. Types of Mutations

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably 1 to 10 residues, and typically are contiguous.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions of from one residue to polypeptides of essentially unrestricted length, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the mature VEGF sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5. An example of a terminal insertion includes a fusion of

a signal sequence, whether heterologous or homologous to the host cell, to the N-terminus of the variant VEGF molecule to facilitate the secretion of variant VEGF from recombinant hosts.

The third group of variants are those in which at least one amino acid residue in the VEGF molecule, and preferably only one, has been removed and a different residue inserted in its place. Such substitutions preferably are made in accordance with the following Table 1 when it is desired to modulate finely the characteristics of a VEGF molecule or variant thereof.

Table 1

Original Residue	Exemplary Substitutions
Ala (A)	gly; ser
Arg (R)	lys
Asn (N)	gln; his
Asp (D)	glu
Cys (C)	ser
Gln (Q)	asn
Glu (E)	asp
Gly (G)	ala; pro
His (H)	asn; gln
Ile (I)	leu; val
Leu (L)	ile; val
Lys (K)	arg; gln; glu
Met (M)	leu; tyr; ile
Phe (F)	met; leu; tyr
Ser (S)	thr
Thr (T)	ser
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table I, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes in biological properties will be those in which (a) glycine and/or proline is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl,

phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, e.g., glutamyl or aspartyl; (e) a residue having an electronegative side chain is substituted for (or by) a residue having an electropositive charge; or (f) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having such a side chain, e.g., glycine.

Most deletions and insertions, and substitutions in particular, are not expected to produce radical changes in the characteristics of the VEGF molecule or variant thereof. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a variant typically is made by site-specific mutagenesis of the native VEGF-encoding nucleic acid, expression of the variant nucleic acid in recombinant cell culture, and, optionally, purification from the cell culture, for example, by immunoaffinity adsorption on a rabbit polyclonal anti-VEGF column (to absorb the variant by binding it to at least one remaining immune epitope).

Since VEGF tends to aggregate into dimers, it is within the scope hereof to provide hetero- and homodimers, wherein one or both subunits are variants. Where both subunits are variants, the changes in amino acid sequence can be the same or different for each subunit chain. Heterodimers are readily produced by cotransforming host cells with DNA encoding both subunits and, if necessary, purifying the desired heterodimer, or by separately synthesizing the subunits, dissociating the subunits (e.g., by treatment with a chaotropic agent such as urea, guanidine hydrochloride, or the like), mixing the dissociated subunits, and then reassociating the subunits by dialyzing away the chaotropic agent.

Also included within the scope of mutants herein are so-called glyco-scan mutants. This embodiment takes advantage of the knowledge of so-called glycosylation sites which are identified by the sequence - NX(S/T) wherein N represents the amino acid asparagine, X represents any amino acid except proline and probably glycine and the third position can be occupied by either amino acid serine or threonine. Thus, where appropriate, such a glycosylation site can be introduced so as to produce a species containing glycosylation moieties at that position. Similarly, an existing glycosylation site can be removed by mutation so as to produce a species that is devoid of glycosylation at that site. It will be understood, again, as with the other mutations contemplated by the present invention, that they are introduced at amino acid position(s) 51 and/or 60 of the native VEGF amino acid sequence in accord with the basic premise of the present invention, and they can be introduced at other locations outside of these amino acid positions within the overall molecule so long as the final product does not differ in overall kind from the properties of the original VEGF variant.

The activity of the cell lysate or purified VEGF variant is then screened in a suitable screening assay for the desired characteristic. For example, binding to the cell-surface VEGF receptor can be routinely assayed by employing well known VEGF binding assays such as those described in the Examples below. A change in the immunological character of the VEGF molecule, such as affinity for a given antibody, is measured by a competitive-type immunoassay. Changes in the enhancement or suppression of vascular endothelium growth by the candidate variants are measured by the appropriate assay (see Examples below). Modifications of such protein properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

3. Recombinant Expression

The variant VEGF molecule desired may be prepared by any technique, including by recombinant methods. Likewise, an isolated DNA is understood herein to mean chemically synthesized DNA, cDNA, chromosomal, or extrachromosomal DNA with or without the 3'- and/or 5'-flanking regions. Preferably, the desired VEGF variant herein is made by synthesis in recombinant cell culture.

For such synthesis, it is first necessary to secure nucleic acid that encodes a VEGF molecule. DNA encoding a VEGF molecule may be obtained from bovine pituitary follicular cells by (a) preparing a cDNA library from these cells, (b) conducting hybridization analysis with labeled DNA encoding the VEGF or fragments thereof (up to or more than 100 base pairs in length) to detect clones in the library containing homologous sequences, and (c) analyzing the clones by restriction enzyme analysis and nucleic acid sequencing to identify full-length clones. DNA encoding a VEGF molecule from a mammal other than bovine can also be obtained in a similar fashion by screening endothelial or leukemia cell libraries. DNA that is capable of hybridizing to a VEGF-encoding DNA under low stringency conditions is useful for identifying DNA encoding VEGF. Both high and low stringency conditions are defined further below. If full-length clones are not present in a cDNA library, then appropriate fragments may be recovered from the various clones using the nucleic acid sequence information disclosed herein for the first time and ligated at restriction sites common to the clones to assemble a full-length clone encoding the VEGF molecule. Alternatively, genomic libraries will provide the desired DNA.

Once this DNA has been identified and isolated from the library it is ligated into a replicable vector for further cloning or for expression.

In one example of a recombinant expression system a VEGF-encoding gene is expressed in mammalian cells by transformation with an expression vector comprising DNA encoding the VEGF. It is preferable to transform

host cells capable of accomplishing such processing so as to obtain the VEGF in the culture medium or periplasm of the host cell, i.e., obtain a secreted molecule.

a. Useful Host Cells and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and construction of the vectors useful in the invention. For example, E. coli K12 strain MM 294 (ATCC No. 31,446) is particularly useful. Other microbial strains that may be used include E. coli strains such as E. coli B and E. coli X1776 (ATCC No. 31,537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as E. coli strains W3110 (F-, lambda-, prototrophic, ATCC No. 27,325), K5772 (ATCC No. 53,635), and SR101, bacilli such as Bacillus subtilis, and other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various pseudomonas species, may be used.

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species (see, e.g., Bolivar *et al., Gene 2*, 95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of its own proteins.

Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang *et al., Nature, 375*, 615 [1978]; Itakura *et al., Science, 198*, 1056 [1977]; Goeddel *et al., Nature, 281*, 544 [1979]) and a tryptophan (trp) promoter system (Goeddel *et al., Nucleic Acids Res., 8*, 4057 [1980]; EPO Appl. Publ. No. 0036,776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (see, e.g., Siebenlist *et al., Cell, 20*, 269 [1980]).

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures, may also be used. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example (Stinchcomb *et al., Nature 282*, 39 [1979]; Kingsman *et al., Gene 7*, 141 [1979]; Tschemper *et al., Gene 10*, 157 [1980]), is commonly used. This plasmid already contains the trp1 gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44,076 or PEP4-1 (Jones, *Genetics, 85*, 12 [1977]). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al., J. Biol. Chem. 255*, 2073 [1980]) or other glycolytic enzymes (Hess *et al., J. Adv. Enzyme Reg. 7*, 149 [1968]; Holland *et al., Biochemistry 17*, 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing

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suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication [Fiers *et al., Nature, 273,* 113 (1978)]. Smaller or larger SV40 fragments may also

be used, provided there is included the approximately 250-bp sequence extending from the HindIII site toward the BglI site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Satisfactory amounts of protein are produced by cell cultures; however, refinements, using a secondary coding sequence, serve to enhance production levels even further. One secondary coding sequence comprises dihydrofolate reductase (DHFR) that is affected by an externally controlled parameter, such as methotrexate (MTX), thus permitting control of expression by control of the methotrexate concentration.

In selecting a preferred host cell for transfection by the vectors of the invention that comprise DNA sequences encoding both VEGF and DHFR protein, it is appropriate to select the host according to the type of DHFR protein employed. If wild-type DHFR protein is employed, it is preferable to select a host cell that is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for successful transfection in selective medium that lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. (USA)* 77, 4216 (1980).

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it is not necessary to use DHFR-deficient cells. Because the mutant DHFR is resistant to methotrexate, MTX-containing media can be used as a means of selection provided that the host cells are themselves methotrexate sensitive. Most eukaryotic cells that are capable of absorbing MTX appear to be methotrexate sensitive. One such useful cell line is a CHO line, CHO-K1 (ATCC No. CCL 61).

b. Typical Methodology Employable

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to prepare the plasmids required.

If blunt ends are required, the preparation may be treated for 15 minutes at 15°C with 10 units of Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments may be performed using 6 percent polyacrylamide gel described by Goeddel *et al., Nucleic Acids Res. 8*, 4057 (1980).

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are typically used to transform E. coli K12 strain 294 (ATCC 31,446) or other suitable E. coli strains, and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared and analyzed by restriction mapping and/or DNA sequencing by the method of Messing *et al., Nucleic Acids Res. 9*, 309 (1981) or by the method of Maxam *et al., Methods of Enzymology 65*, 499 (1980).

After introduction of the DNA into the mammalian cell host and selection in medium for stable transfectants, amplification of DHFR-protein-coding sequences is effected by growing host cell cultures in the presence of approximately 20,000-500,000 nM concentrations of methotrexate, a competitive inhibitor of DHFR activity. The effective range of concentration is highly dependent, of course, upon the nature of the DHFR gene and the characteristics of the host. Clearly, generally defined upper and lower limits cannot be ascertained. Suitable concentrations of other folic acid analogs or other compounds that inhibit DHFR could also be used. MTX itself is, however, convenient, readily available, and effective.

Other techniques employable are described in a section just prior to the examples.

4. Utilities and Formulation

The variant VEGF antagonists of the present invention have a number of therapeutic uses associated with the vascular endothelium. Such uses include, for example, incorporation into formed articles which can be used in modulating endothelial cell growth and angiogenesis. In addition, tumor invasion and metastasis may be modulated with these articles. Other disorders for which the polypeptides of the present invention may find use are discussed supra.

For the indications referred to above, the variant VEGF antagonist molecule will be formulated and dosed in a fashion consistent with good medical practice taking into account the specific disease or disorder to be treated, the condition of the individual patient, the site of delivery of the VEGF antagonist, the method of administration, and other factors known to practitioners. Thus, for purposes herein, the "therapeutically effective amount" of the VEGF is an amount that is effective either to prevent, lessen the worsening of, alleviate, or cure the treated condition, in particular that amount which is sufficient to substantially inhibit the growth of vascular endothelium *in vivo*.

VEGF amino acid sequence variants and derivatives that are immunologically crossreactive with antibodies raised against native VEGF are useful in immunoassays for VEGF as standards, or, when labeled, as competitive reagents.

The VEGF antagonist is prepared for storage or administration by mixing VEGF antagonist having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to recipients at the dosages and concentrations employed. If the VEGF antagonist is water soluble, it may be formulated in a buffer such as phosphate or other organic acid salt preferably at a pH of about 7 to 8. If a VEGF variant is only partially soluble in water, it may be prepared as a microemulsion by formulating it with a nonionic surfactant such as Tween, Pluronics, or PEG, e.g., Tween 80, in an amount of 0.04-0.05% (w/v), to increase its solubility.

Optionally other ingredients may be added such as antioxidants, e.g., ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; and sugar alcohols such as mannitol or sorbitol.

The VEGF antagonist to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). The VEGF ordinarily will be stored in lyophilized form or as an aqueous solution if it is highly stable to thermal and oxidative denaturation. The pH of the VEGF antagonist preparations typically will be about from 6 to 8, although higher or lower pH values may also be appropriate in certain instances. It

will be understood that use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of salts of the VEGF antagonist.

If the VEGF antagonist is to be used parenterally, therapeutic compositions containing the VEGF antagonist generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Generally, where the disorder permits, one should formulate and dose the VEGF for site-specific delivery. This is convenient in the case of site-specific solid tumors.

Sustained release formulations may also be prepared, and include the formation of microcapsular particles and implantable articles. For preparing sustained-release VEGF antagonist compositions, the VEGF antagonist is preferably incorporated into a biodegradable matrix or microcapsule. A suitable material for this purpose is a polylactide, although other polymers of poly-(a-hydroxycarboxylic acids), such as poly-D-(-)-3-hydroxybutyric acid (EP 133,988A), can be used. Other biodegradable polymers include poly(lactones), poly(acetals), poly(orthoesters), or poly(orthocarbonates). The initial consideration here must be that the carrier itself, or its degradation products, is nontoxic in the target tissue and will not further aggravate the condition. This can be determined by routine screening in animal models of the target disorder or, if such models are unavailable, in normal animals. Numerous scientific publications document such animal models.

For examples of sustained release compositions, see U.S. Patent No. 3,773,919, EP 58,481A, U.S. Patent No. 3,887,699, EP 158,277A, Canadian Patent No. 1176565, U. Sidman *et al., Biopolymers 22*, 547 [1983], and R. Langer *et al., Chem. Tech. 12*, 98 [1982].

When applied topically, the VEGF antagonist is suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable and efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal patches, plasters, and bandages, preferably in liquid or semi-liquid form.

For obtaining a gel formulation, the VEGF antagonist formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as polyethylene glycol to form a gel of the proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullullan; agarose; carrageenan; dextrans; dextrins; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the VEGF antagonist held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified, and listed in USP, e.g., methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight polyethylene glycols to obtain the proper viscosity. For example, a mixture of a polyethylene glycol of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The term "water soluble" as applied to the polysaccharides and polyethylene glycols is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

If methylcellulose is employed in the gel, preferably it comprises about 2-5%, more preferably about 3%, of the gel and the VEGF antagonist is present in an amount of about 300-1000 mg per ml of gel.

The dosage to be employed is dependent upon the factors described above. As a general proposition, the VEGF antagonist is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a VEGF antagonist level greater than about 0.1 ng/cc up to a maximum dose that is efficacious but not unduly toxic. This intra-tissue concentration should be maintained if possible by continuous infusion, sustained release, topical application, or injection at empirically determined frequencies.

5. Pharmaceutical Compositions

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby

desirable to provide somewhat larger initial doses, such as an intravenous bolus.

For the various therapeutic indications referred to for the compounds hereof, the VEGF antagonists will be formulated and dosed in a fashion consistent with good medical practice taking into account the specific disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners in the respective art. Thus, for purposes herein, the "therapeutically effective amount" of the VEGF molecules hereof is an amount that is effective either to prevent, lessen the worsening of, alleviate, or cure the treated condition, in particular that amount which is sufficient to substantially reduce or inhibit the growth of vascular endothelium *in vivo*. In general a dosage is employed capable of establishing in the tissue that is the target for the therapeutic indication being treated a level of a VEGF antagonist hereof greater than about 0.1 ng/cm^3 up to a maximum dose that is efficacious but not unduly toxic. It is contemplated that intra-tissue administration may be the choice for certain of the therapeutic indications for the compounds hereof.

The following examples are intended merely to illustrate the best mode now known for practicing the invention but the invention is not to be considered as limited to the details of such examples.

EXAMPLE I

Materials - Muta-gene phagemid *in vitro* mutagenesis kit, horse-radish peroxidase conjugated goat IgG specific for murine IgG, pre-stained low-range MW standards and Trans-Blot Transfer Medium (pure nitrocellulose membrane) were purchased from BioRad Laboratories (Richmond, CA). Qiagen plasmid Tip 100 kit and Sequenase version 2.0 were from Qiagen (Chatsworth, CA) and United States Biochemical (Cleveland, OH), respectively. SDS gels (4-20% gradient polyacrylamide) and pre-cut blotting paper were from Integrated Separations Systems

(Natick, MA). SDS sample buffer (x concentrate) and various restriction enzymes were from New England Biolabs (Beverly, MA). O--phenylenediamine, citrate phosphate buffers, sodium dodecyl sulfate, and H_2O_2 substrate tablets were purchased from Sigma (St. Louis, MO). BufferEZE formula 1 (transfer buffer) and X-OMat AR X-ray film were from Eastman Kodak Co. (Rochester, NY). Maxosorb and Immunlon-1 microtiter plates were purchased from Nunc (Kamstrup, Denmark) and Dynatech (Chantilly, VA), respectively. Cell culture plates (12-well) and culture media (with calf serum) were from Costar (Cambridge, MA) and Gibco (Grand Island, NY), respectively. Polyethylene-20-sorbitan monolaurate (Tween-20) was from Fisher Biotech (Fair Lawn, NJ). G25 Sephadex columns (PD-10) and $_{125}$I labeled Protein A were from Pharmacia (Piscataway, NJ) and Amersham (Arlington Heights, IL), respectively. Bovine serum albumin (BSA) and rabbit IgG anti-human IgG (Fc-specific) were purchased from Cappel (Durham, NC) and Calbiochem (La Jolla, CA), respectively. Plasmid vector (pRK5), competent *E. coli* cells (DH5a and CJ236), synthetic oligonucleotides, cell culture medium, purified CHO-derived $VEGF_{165}$, monoclonal (Mates A4.6.1, 2E3, 4D7, SC3, and SF8) and polyclonal antibodies to $VEGF_{165}$ were prepared at Genentech, Inc. (South San Francisco, CA). Construction, expression and purification of FLT-1, flkl and KDR receptor-IgG chimeras was as described by Park, *et al. J. Biol. Chem. 269*, 25646-25654 (1994).

Site-directed Mutagenesis and Expression of VEGF Variants - Site-directed mutagenesis was performed using the Muta-Gene Phagemid *in vitro* mutagenesis kit according to the method of Kunkel *Proc. Natl. Acad. Sci. 82*, 488-492 (1985) and Kunkel *et al., Methods Enzymol. 154*, 367-382 (1987). A plasmid vector pRK5 containing cDNA for $VEGF_{165}$ isoform was used for mutagenesis and transient expression. The pRK5 vector is a modified pUC118 vector and contains a CMV enhancer and promoter [Nakamaye *et al., Nucleic Acids Res. 14*, 9679-9698 (1986) and Vieira *et al., Methods Enzymol. 155*, 3-11 (1987)]. The mutagenized DNA was purified using the Qiagen Plasmid Midi Kit Tip 100 and the sequence of

the mutations was verified using Sequenase Version 2.0 Kit. The mutated DNA was analyzed by restriction enzyme digestion as described by Sambrook, *et al., Molecular Cloning: A Laboratory Manual* part I, C5.28-5.32, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Transient transfection of human fetal kidney "293 cells" was performed in 6-well plates using the modified calcium phosphate precipitate method as previously described [Jordan *et al., Bio/Technology (manuscript in preparation)* (1994); Chen *et al., Mol. Cell. Biol. 7*, 2745-2752 (1987); Gorman *et al., DNA and Protein Engineering Techniques 2*, 3-10 (1990); Graham *et al., Virology 52*, 456-467 (1973)]. Briefly, approximately 1.2 x 10^6 cells were incubated overnight at 37°C in the presence of 15 μg of precipitated DNA. Cell culture supernatant was replaced with serum free medium, and cell monolayers were incubated for 72 hours at 37°C. Conditioned media (3 ml) was harvested, centrifuged, aliquoted and stored at -70°C until use.

Quantitation of $VEGF_{165}$ Variants by ELISA - A radioimmunometric assay previously described [Aiello *et al., N. Engl. J. Med. 331*, 1480-1487 (1994)], was adapted for the quantitation of VEGF mutants by the following procedure. Individual wells of a 96-well microtiter plate were coated with 100 μl of a 3 μg/ml solution of an anti-$VEGF_{165}$ polyclonal antibody in 50 mM sodium carbonate buffer pH 9.6 overnight at 4°C. The supernatant was discarded, and the wells were washed 4 times with PBS containing 0.03% Tween 80. The plate was blocked in assay buffer (0.5% BSA, 0.03% Tween 80, 0.01% Thimerosal in PBS) for one hr (300 μl/well) at ambient temperature, then the wells were washed. Diluted samples (100 μl) and $VEGF_{165}$ standard (ranging from 0.1 to 10 ng/ml) were added to each well and incubated for one hr at ambient temperature with gentle agitation. The supernatant was discarded, and the wells were washed. Anti-VEGF murine monoclonal antibody 5F8 solution (100 μl at 1 μg/ml) was added, and the microtiter plate was incubated at ambient

temperature for one hr with gentle agitation. After the supernatant was discarded, the plate was washed and horseradish peroxidase conjugated goat IgG specific for murine IgG (100 µl) at a dilution of 1:25000 was immediately added to each well. The plate was incubated for one hr at ambient temperature with gentle agitation after which the supernatant discarded, the wells washed, and developed by addition of ortho-phenylenediamine (0-04%), H_2O_2 (0.012%) in 50 mM citrate phosphate buffer pH 5 (100 µl), then incubated in the dark at ambient temperature for 10 min. The reaction was stopped by adding 50 µl of 4.5 N H_2SO_4 to each well and the absorbance was measured at 492 nm on a microplate reader (SLT Labs). The concentrations of $VEGF_{165}$ variants were quantitated by interpolation of a standard curve using non-linear regression analysis. For purposes of comparison, a second ELISA was developed that utilized a dual monoclonal format. The assay was similar to the above described ELISA, except a neutralizing monoclonal antibody (Mab A4.6.1) was used to coat the microtiter plates [Kim *et al., Growth Factors* 7 , 53-64 (1992)].

Immunoblotting of VEGF mutants - Aliquots of conditioned cell media (16 µl) containing VEGF or VEGF mutant (approx. 10 ng) were added to x SDS sample buffer (4 µl) and heated at 90°C for 3 min prior to loading on SDS polyacrylamide (4 to 20% acrylamide) gels. Pre-stained MW standards (10 µl) were loaded in the outer lanes of the SDS gels. Gels were run at 25 mA for 90 min at 4°C. Gels were transferred to nitrocellulose paper in a Bio-Rad tank blotter containing BufferEZE with 0.1% SDS for 90 min at 250 mA at 25°C. Nitrocellulose was pre-wetted in transfer buffer with 0.1% SDS for 10 min prior to use. Transferred immunoblots were blocked in PBS overnight with 1.0% BSA and 0.1% Tween 20 (blocking buffer) at 4°C. A solution containing 5 murine anti-VEGF Mabs (A.4.6.1, 5C3, 5F8, 4D7, and 2E3) was prepared with 2 µg/ml of each Mab in blocking buffer and used as primary antibody. The primary antibody solution was incubated with the immunoblots for 4 hr at 25°C with gentle agitation, then washed 3x for 10 min in blocking buffer at 25°C. ^{125}I labeled Protein

A was diluted to 10^4 cpm/ml (final concentration) in blocking buffer and incubated with the immunoblots for 60 min with gentle agitation at 25°C. Immunoblots were washed 3x for 10 min in blocking buffer at 25°C, then dried on filter paper and placed on Kodak X-Omat film with two intensifying screens at -70°C for 3 days.

Preparation of ^{125}I labeled $VEGF_{165}$ - Radiolabeling of CHO-derived $VEGF_{165}$ was prepared using a modification of the chloramine T catalyzed iodination method [Hunter *et al., Nature 194*, 495-496 (1962)] . In a typical reaction, 10 µl of 1 M Tris-HCl, 0.01% Tween 20 at pH 7.5 was added to 5 µl of sodium iodide-125 (0.5 milliCuries, 0.24 nmol) in a capped reaction vessel. To this reaction, 10 µl of CHO-derived $VEGF_{165}$ (10 µg, 0.26 nmol) was added. The iodination was initiated by addition of 10 µl of 1 mg/ml chloramine T in 0.1 M sodium phosphate, pH 7.4. After 60 sec, iodination was terminated by addition of sodium metabisulfite (20 µl, 1 mg/ml) in 0.1 M sodium phosphate, pH 7.5. The reaction vessel was vortexed after each addition. The reaction mixture was applied to a PD-10 column (G25 Sephadex) that was pre-equilibrated with 0.5% BSA, 0.01% Tween 20 in PBS. Fractions were collected and counted for radioactivity with a gamma scintillation counter (LKB model 1277). Typically, the specific radioactivity of the iodinated VEGF was 26 ± 2.5 µCi/µg, which corresponded to one ^{125}I per two molecules of $VEGF_{165}$ dimer.

$VEGF_{165}$ Receptor Binding Assay - The assay was performed in 96-well immunoplates (Immulon-1); each well was coated with 100 µl of a solution containing 10 µg/ml of rabbit IgG anti-human IgG (Fc-specific) in 50 mM sodium carbonate buffer pH 9.6 overnight at 4°C. After the supernatant was discarded, the wells were washed three times in washing buffer (0.01% Tween 80 in PBS). The plate was blocked (300 µl/well) for one hr in assay buffer (0.5% BSA, 0.03% Tween 80, 0.01% Thimerosal in PBS). The supernatant was discarded and the wells were washed. A cocktail was prepared with conditioned cell media containing $VEGF_{165}$ mutants at varying concentrations (100 µl), ^{125}I radiolabeled $VEGF_{165}$

(approx. $5x10_3$ cpm in 50 μl) which was mixed with VEGF receptor-IgG chimeric protein, FLT-1 IgG, flk-1 IgG or KDR-IgG (3-15 ng/ml, final concentration, 50 μl) in micronic tubes. Aliquots of this solution (100 μl) were added to pre-coated microtiter plates and incubated for 4 hr at ambient temperature with gentle agitation. The supernatant was discarded, the plate washed, and individual microtiter wells were counted by gamma scintigraphy (LKB model 1277). The competitive binding between unlabeled $VEGF_{165}$ (or $VEGF_{165}$ mutants) and ^{125}I radiolabeled $VEGF_{165}$ to the FLT-1, Flk-1, or KDR receptors were plotted, and analyzed using a four parameter fitting program (Kaleidagraph, Adelbeck Software). The apparent dissociation constant for each VEGF mutant was estimated from the concentration required to achieve 50% inhibition (IC_{50}).

Assay for Vascular Endothelial Cell Growth - The mitogenic activity of VEGF variants was determined by using bovine adrenal cortical endothelial (ACE) cells as target cells as previously described [Ferrara *et al., Biochem. Biophys. Res. Comm. 161*, 851-859 (1989)]. Briefly, cells were plated sparsely (7000 cells/well) in 12 well plates and incubated overnight in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, and antibiotics. The medium was exchanged the next day, and VEGF or VEGF mutants, diluted in culture media at concentrations ranging from 100 ng/ml to 10 pg/ml, were layered in duplicate onto the seeded cells. After incubation for 5 days at 37°C, the cells were dissociated with trypsin, and quantified using a Coulter counter.

Isolation of VEGF cDNA

Total RNA was extracted [Ullrich *et al., Science 196*, 1313-1317 (1977)] from bovine pituitary follicular cells [obtained as described by Ferrara *et al., Meth. Enzymol.* supra, and Ferrara *et al., Am. J. Physiol.*, supra] and the polyadenylated mRNA fraction was isolated by oligo(dT)-cellulose chromatography. Aviv *et al., Proc. Natl. Acad. Sci. USA 69*, 1408-1412 (1972). The cDNA was prepared [Wickens *et al., J. Biol. Chem. 253*, 2483-2495 (1978)] by priming with dT_{12-18} or a random hexamer dN_6.

The double-stranded cDNA was synthesized using a cDNA kit from Amersham, and the resulting cDNA was subcloned into EcoRI-cleaved lgt10 as described [Huynh *et al., DNA Cloning Techniques, A Practical Approach*, Glover ed. (IRL, Oxford, 1985)], except that asymmetric EcoRI linkers [Norris *et al., Gene 7*, 355-362 (1979)] were used, thus avoiding the need for the EcoRI methylase treatment.

The recombinant phage were plated on E. coli C600 Hfl [Huynh *et al.* supra] and replica plated onto nitrocellulose filters. Benton *et al., Science 196*, 180-182 (1977). These replica were hybridized with a ^{32}P-labeled [Taylor *et al., Biochim. Biophys. Acta, 442*, 324-330 (1976)] synthetic oligonucleotide probe of the sequence:

5'- CCTATGGCTGAAGGCGGCCAGAAGCCTCACGAAGTGGTGAAGTTCATGGACGTGTATCA-3'

at 42°C in 20% formamide, 5 x SSC, 50 mM sodium phosphate pH 6.8, 0.1% sodium pyrophosphate, 5 x Denhardt's solution, and 50 mg/ml salmon sperm DNA, and washed in 2 x SSC, 0.1% SDS at 42°C.

One positive clone, designated l.vegf.6, was identified. This clone, labeled with ^{32}P, was used as a probe to screen an oligo-dT-primed human placenta cDNA library, and positive clones were observed. When a human pituitary cDNA library was screened with the same labeled clone, no positive clones were detected.

The complete nucleotide sequence of the clone l.vegf.6 was determined by the dideoxyoligonucleotide chain termination method [Sanger *et al., Proc. Natl. Acad. Sci. USA 74*, 5463-5467 (1977)] after subcloning into the pRK5 vector. The sequence obtained, along with the imputed amino acid sequence, including the signal sequence.

Expression of VEGF-Encoding Gene in Mammalian Cells

The final expression vector, pRK5.vegf.6, was constructed from l.vegf.6 and pRK5. The construction of pRK5 and pRK5.vegf.6 is described below in detail.

```
 1   5' AGTAGCAAGCTTGACGTGTGGCAGGCTTGA...
31     GATCTGGCCATACACTTGAGTGACAATGA...
60     CATCCACTTTGCCTTTCTCTCCACAGGT...
88     GTCCACTCCCAG 3'
 1   3' CAGGTGAGGGTGCAGCTTGACGTCGTCGGA 5'
```

DNA polymerase I (Klenow fragment) filled in the synthetic piece and created a double-stranded fragment. Wartell, R.M. and W.S. Reznikoff, *Gene, 9*, 307 (1980). This was followed by a double digest of PstI and HindIII. This synthetic linker was cloned into pUC13 (Veira and Messing, op. cit.) at the PstI and HindIII sites. The clones containing the synthetic oligonucleotide, labeled pUCIg.10, was digested with PstI. A ClaI site was added to this fragment by use of a PstI-ClaI linker. Following digestion with HindIII a 118-bp piece containing part of the Ig intron and the Ig variable region splice acceptor was gel isolated.

3) The third part of the construction scheme replaced the hepatitis surface antigen 3' end with the polyadenylation site and transcription termination site of the early region of SV40. A vector, pUC.SV40, containing the SV40 sequences was inserted into pUC8 at the BamHI site described by Vieira and Messing, op. cit. pUC.SV40 was then digested with EcoRI and HpaI. A 143bp fragment containing the SV40 polyadenylation sequence was gel isolated from this digest. Two additional fragments were gel isolated following digestion of pSVE.8c1D. (European Pat. Pub. No. 160,457). The 4.8 kb fragment generated by EcoRI and Cla1 digestion contains the SV40-DHFR transcription unit, the origin of replication of pML and the ampicillin resistance marker. The 7.5-kb fragment produced following digestion with ClaI and HpaI contains the cDNA for Factor VIII. A three-part ligation yielded pSVE.8c24D. This intermediate plasmid was digested by ClaI and SalI to give a 9611 bp fragment containing the cDNA for Factor VIII with an SV40 poly A site followed by the SV40 DHFR transcription unit.

was isolated, and 3) the 5828 bp XbaI-ClaI BAP fragment of pCIS2.8c24D. The translated DNA sequence of the resultant variant in the exact fusion junction region of pCIS2.8c28D was determined and correlates.

A.3. Construction of pRK5

The starting plasmid for construction of pRK5 was pCIS2.8c28D. The base numbers in paragraphs 1 through 6 refer to pCIS2.8c28D with base one of the first T of the EcoRI site preceding the CMV promoter. The cytomegalovirus early promoter and intron and the SV40 origin and polyA signal were placed on separate plasmids.

1. The cytomegalovirus early promoter was cloned as an EcoRI fragment from pCIS2.8c28D (9999-1201) into the EcoRI site of pUC118 described above. Twelve colonies were picked and screened for the orientation in which single-stranded DNA made from pUC118 would allow for the sequencing from the EcoRI site at 1201 to the EcoRI site at 9999. This clone was named pCMVE/P.

2. Single-stranded DNA was made from pCMVE/P in order to insert an SP6 (Green, MR *et al., Cell 32*, 681-694 [1983]) promoter by site-directed mutagenesis. A synthetic 110 mer that contained the sequences from -69 to +5 of SP6 promoter (see *Nucleic Acids Res., 12*, 7041 [1984]) were used along with 18-bp fragments on either end of the oligomer corresponding to the CMVE/P sequences. Mutagenesis was done by standard techniques and screened using a labeled 110 mer at high and low stringency. Six potential clones were selected and sequenced. A positive clone was identified and labeled pCMVE/PSP6.

3. The SP6 promoter was checked and shown to be active, for example, by adding SP6 RNA polymerase and checking for RNA of the appropriate size.

4. A ClaNotISma adapter was synthesized to encompass the location from the ClaI site (912) to the SmaI site of pUC118 in pCMVE/P (step 1) and pCMVE/PSP6 (step 2). This adapter was ligated into the ClaI-SmaI site of pUC118 and screened for the correct clones. The linker was sequenced in both and clones were labeled pCMVE/PSP6-L and pCMVE/P-L.

5. pCMVE/PSP6-L was cut with SmaI (at linker/pUC118 junction) and HindIII (in pUC118). A HpaI (5573)-to-HindIII (6136) fragment from pSVORAADRI 11, described below, was inserted into SmaI-HindIII of pCMVE/PSP6-L. This ligation was screened and a clone was isolated and named pCMVE/PSP6-L-SVORAADRI.

a) The SV40 origin and polyA signal was isolated as the XmnI (5475) - HindIII (6136) fragment from pCIS2.8c28D and cloned into the HindIII to SmaI sites of pUC119 (described in Vieira and Messing, op. cit.). This clone was named pSVORAA.

b) The EcoRI site at 5716 was removed by partial digestion with EcoRI and filling in with Klenow. The colonies obtained from self-ligation after fill-in were screened and the correct clone was isolated and named pSVORAADRI 11. The deleted EcoRI site was checked by sequencing and shown to be correct.

c) The HpaI (5573) to HindIII (6136) fragment of pSVORAADRI 11 was isolated and inserted into pCMVE/PSP6-L (see 4 above).

6. pCMVE/PSP6-L-SVOrAADRI (step 5) was cut with EcoRI at 9999, blunted and self-ligated. A clone without an EcoRI site was identified and named pRK.

7. pRK was cut with SmaI and BamHI. This was filled in with Klenow and relegated. The colonies were screened. A positive clone was identified and named pRKDBam/Sma3.

8. The HindIII site of pRKDBam/Sma3 was converted to a HpaI site using a converter. (A converter is a piece of DNA used to change one restriction site to another. In this case one end would be complementary to a HindIII sticky end and the other end would have a recognition site for HpaI.) A positive clone was identified and named pRKDBam/Sma, HIII-HpaI 1.

9. pRKDBam/Sma, HIII-HpaI 1 was cut with PstI and NotI and an EcoRI-HindIII linker and HindIII-EcoRI linker were ligated in. Clones for each linker were found. However, it was also determined that too many of the HpaI converters had gone in (two or more converters generate a PvuII site). Therefore, these clones had to be cut with HpaI and self-ligated.

10. RI-HIII clone 3 and HIII-RI clone 5 were cut with HpaI, diluted, and self-ligated. Positives were identified. The RI-HIII clone was named pRK5.

B. Construction of pRK5.vegf.6

The clone I.vegf.6 was treated with EcoRI and the EcoRI insert was isolated and ligated into the vector fragment of pRK5 obtained by digestion of pRK5 with EcoRI and isolation of the large fragment. The two-part ligation of these fragments yielded the expression vector, pRK5.vegf.6, which was screened for the correct orientation of the VEGF-encoding sequence with respect to the promoter.

Further details concerning the construction of the basic pRK5 vector can be taken from U.S. Patent 5,332,671 that issued on 26 July 1994, said patent being expressly incorporated herein by reference.

EXAMPLE 2

The following example details the methodology generally employed to prepare the various VEGF mutants covered by the present invention. The basic expression vector was prepared as follows:

Vector $SDVF_{165}$ containing the cDNA of $VEGF_{165}$ was obtained. The cDNA for $VEGF_{165}$ was isolated from $SDVF_{165}$ by restriction digestion with Hind III and Eco RI. This isolated insert was ligated into the pRK5 plasmid taking advantage to the existence therein of Eco RI and Hind III sites. The resultant plasmid was transformed into competent CJ236 E. coli cells to make a template for site-directed mutagenesis. The corresponding oligonucleotide containing the mutated site was then prepared - see infra - and the in vitro site-directed mutagenesis step was conducted in accordance with known procedures using the BioRad Muta-Gene mutagenesis kit. After sequencing to determine that the mutagenized site was incorporated into the final expression vector, the resultant vector was transfected into 293 human kidney cells for transient expression.

The following oligonucleotides were prepared in order to make the final mutated product.

Table 1

Mutation	5' to 3' Sequence
C51D	CAGGGGCACATCGGATGGCTTGAA
C51A	CAGGGGCACGGCGGATGGCTTGAA
C60D	GTCATTGCAATCGCCCCCGCATCG
C60A	GTCATTGCAGGCGCCCCCGCATCG
C51A, C60A	GTCATTGCAGGCGCCCCCGCATCGCATCAGG GGCACGGCGGATGGCTTGAA
C51D, C60D	GTCATTGCAATCGCCCCCGCATCGCATCAGGG GCACATCGGATGGCTTGAA

Thus prepared in accordance with the insertion of the oligonucleotides set forth in Table 1 above, left column there are prepared at the corresponding mutation in the VEGF molecule in accordance with the notation given under the left hand column entitled "Mutation". The naming of the compound is in accord with naming convention. Thus, for the first entry the mutation is referred to as "C51D". This means that at the 51 amino acid position of the

VEGF molecule the cysteine (C) residue was mutated so as to insert an aspartic acid (D) at that 51 position.

Figure 2 is a diagram showing the native VEGF dimer and certain of the variant VEGF polypeptides of the present invention. As shown in Figure 2, the native VEGF molecule dimerizes through the formation of disulfide bonds between the cysteine at amino acid position 51 on one monomer and the cysteine at amino acid position 60 on the other monomer and vice versa. Changing the cysteine residue at amino acid position 51 or 60 to aspartic acid (C51D or C60D, respectively) prevents proper dimerization and the formation of staggered dimer molecules. Changing both cysteine residues at amino acid positions 51 and 60 (C51D, C60D) prevents dimer formation altogether.

Binding of VEGF Variants to VEGF Receptors - Native VEGF dimer and the VEGF variant polypeptides shown in Figure 2 were tested for the ability to bind to the KDR and FLT-1 receptors. Receptor binding assays were performed as described above. The results obtained for binding to the KDR receptor are presented in Figures 3 and 4.

As shown in Figure 3, all of the three VEGF variant polypeptides tested retained the ability to bind to the KDR receptor, although none exhibited a binding affinity as great as the native VEGF dimer protein. The results presented in Figure 3 also demonstrate that the monomeric variant polypeptide C51D, C60D retains the ability to bind to the KDR receptor, however, it does so with a reduced binding affinity as compared to the native dimer or two staggered dimers tested. Figure 4 demonstrates that the binding affinity of the C51D, C60D monomeric variant for the KDR receptor is approximately 500-fold less than the native dimeric VEGF protein. Thus, these results demonstrate that each of the VEGF variant polypeptides tested retain the ability to bind to the KDR receptor, although at a lower binding affinity.

Ability of the C51D, C60D Monomer to Inhibit VEGF-Induced Endothelial Cell Growth - The C51D, C60D monomer polypeptide was employed in assays designed to measure the ability of the monomer to inhibit the VEGF-induced growth of endothelial cells. Briefly, endothelial cells were cultured in the presence of 3 ng/ml VEGF and varying amounts of either the A461 anti-VEGF monoclonal antibody or the C51D, C60D monomer polypeptide. The results demonstrating the inhibitory effects of each inhibitor on endothelial cell growth are presented in Figure 8.

The results presented in Figure 8 demonstrate that both the A461 anti-VEGF monoclonal antibody and the C51D, C60D monomer polypeptide exhibit substantial inhibitory effects on VEGF-induced endothelial cell growth. These inhibitory effects increase as the ratio of inhibitor to VEGF increases. As such, the C51D, C60D monomer polypeptide functions to inhibit the endothelial growth activating effect of VEGF.

Concluding Remarks:

The foregoing description details specific methods which can be employed to practice the present invention. Having detailed such specific methods, those skilled in the art will well enough know how to devise alternative reliable methods at arriving at the same information in using the fruits of the present invention. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope thereof; rather, the ambit of the present invention is to be determined only by the lawful construction of the appended claims. All documents cited herein are hereby expressly incorporated by reference.

WHAT IS CLAIMED IS:

1. A VEGF antagonist molecule comprising a variant vascular endothelial growth factor polypeptide, said variant polypeptide comprising an amino acid modification of at least one cysteine residue, wherein said amino acid modification inhibits the ability of said variant polypeptide to properly dimerize with another vascular endothelial growth factor polypeptide monomer, wherein said antagonist molecule is capable of binding to vascular endothelial growth factor receptors without significantly inducing a vascular endothelial growth factor response, and functional derivatives of said antagonist molecule.

2. The antagonist molecule according to Claim 1 wherein said amino acid modification is a substitution of said at least one cysteine residue with a different amino acid which is incapable of participating in a disulfide bond.

3. The antagonist molecule according to Claim 2 wherein said substitution is of the cysteine residue at amino acid position 51 and/or 60 of the native VEGF amino acid sequence.

4. The antagonist molecule according to Claim 3 wherein aspartic acid is substituted for cysteine.

5. The antagonist molecule according to Claim 4 comprising the substitution C51D.

6. The antagonist molecule according to Claim 4 comprising the substitution C60D.

7. The antagonist molecule according to Claim 1 wherein said amino acid modification is a chemical modification of said at least one cysteine residue which renders said cysteine residue incapable of participating in a disulfide bond.

8. The antagonist molecule according to Claim 7 wherein said chemical modification is of the cysteine residue at amino acid position 51 and/or 60 of the native VEGF amino acid sequence.

9. The antagonist molecule according to Claim 1 containing further amino acid modifications that do not otherwise affect the essential biological characteristics.

10. An isolated nucleic acid sequence comprising a sequence that encodes the VEGF antagonist molecule of Claim 1.

11. A replicable expression vector capable in a transformant host cell of expressing the nucleic acid of Claim 10.

12. Host cells transformed with the vector according to Claim 11.

13. Host cells according to Claim 12 which are Chinese hamster ovary cells.

14. A composition of matter comprising the VEGF antagonist molecule according to Claim 1 compounded with a pharmaceutically acceptable carrier.

15. A method of treatment which comprises administering a composition according to Claim 14.

```
  1   CAGTGTGCTG GCGGCCCGGC GCGAGCCGGC CCGGCCCCGG TCGGGCCTCC
-26

      GAAACC    ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC
                M   N   F   L   L   S   W   V   H   W   S
               -26                       -20

 90   CTC GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG
-15   L   A   L   L   L   Y   L   H   H   A   K   W   S   Q
                          -10

      GCT GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC
      A   A   P   M   A   E   G   G   G   Q   N   H   H
      -1  +1              +5                  +10

171   GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC
 13   E   V   V   K   F   M   D   V   Y   Q   R   S   Y   C
        +15                       +20                   +25

      CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC
      H   P   I   E   T   L   V   D   I   F   Q   E   Y
                +30                   +35

252   CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC
 40   P   D   E   I   E   Y   I   F   K   P   S   C   V   P
     +40                   +45                   +50

      CTG ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG
      L   M   R   C   G   G   C   C   N   D   E   G   L
        +55                   +60                   +65

333   GAG TGT GTG CCC ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT
 67   E   C   V   P   T   E   E   S   N   I   T   M   Q   I
                +70                   +75                   +80

      ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC ATA GGA GAG
      M   R   I   K   P   H   Q   G   Q   H   I   G   E
                    +85                   +90

414   ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AGA CCA AAG
 94   M   S   F   L   Q   H   N   K   C   E   C   R   P   K
        +95                   +100                  +105

      AAA GAT AGA GCA AGA CAA GAA AAT CCC TGT GGG CCT TGC
      K   D   R   A   R   Q   E   N   P   C   G   P   C
            +110                  +115                  +120

495   TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG
121   S   E   R   R   K   H   L   F   V   Q   D   P   Q   T
                    +125                  +130

      TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG
      C   K   C   S   C   K   N   T   D   S   R   C   K
    +135                  +140                  +145
```

FIG._1A

```
576   GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC
148   A   R   Q   L   E   L   N   E   R   T   C   R   C   D
          +150                +155                +160

      AAG CCG AGG CGG TGA GCCGGGCA GGAGGAAGGA GCCTCCCTCA
      K   P   R   R   O
              +165

661   GGGTTTCGGG AACCAGATCT CTCACCAGGA AAGACTGATA CAGAACGATC

      GATACAGAAA CCACGCTGCC GCCACCACAC CATCACCATC GACAGAACAG

761   TCCTTAATCC AGAAACCTGA AATGAAGGAA GAGGAGACTC TGCGCAGAGC

      ACTTTGGGTC CGGAGGGCGA GACTCCGGCG GAAGCATTCC CGGGCGGGTG

861   ACCCAGCACG GTCCCTCTTG GAATTGGATT CGCCATTTTA TTTTTCTTGC

      TGCTAAATCA CCGAGCCCGG AAGATTAGAG AGTTTTATTT CTGGGATTCC

961   TGTAGACACA CCGCGGCCGC CAGCACACTG
```

FIG._1B

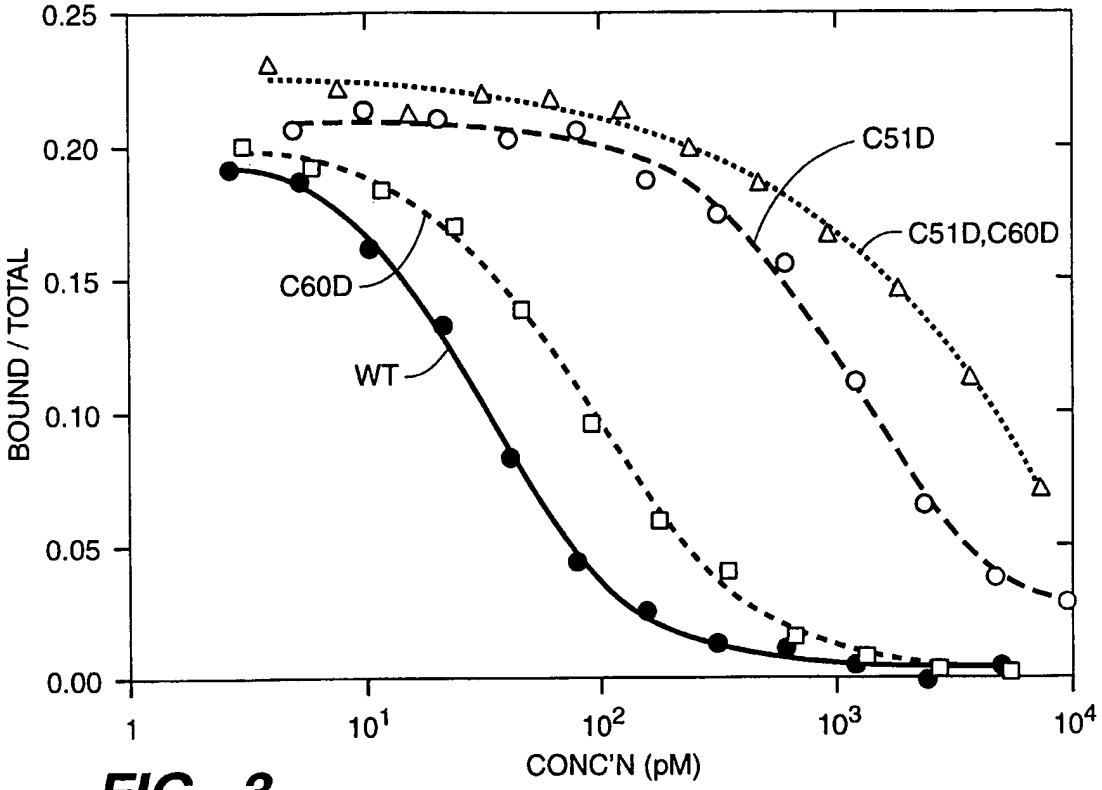

0.25
0.20
0.15
0.10
0.05
0.00
BOUND / TOTAL
C51D
C51D,C60D
C60D
WT
1
10^1
10^2
10^3
10^4
CONC'N (pM)

FIG._3

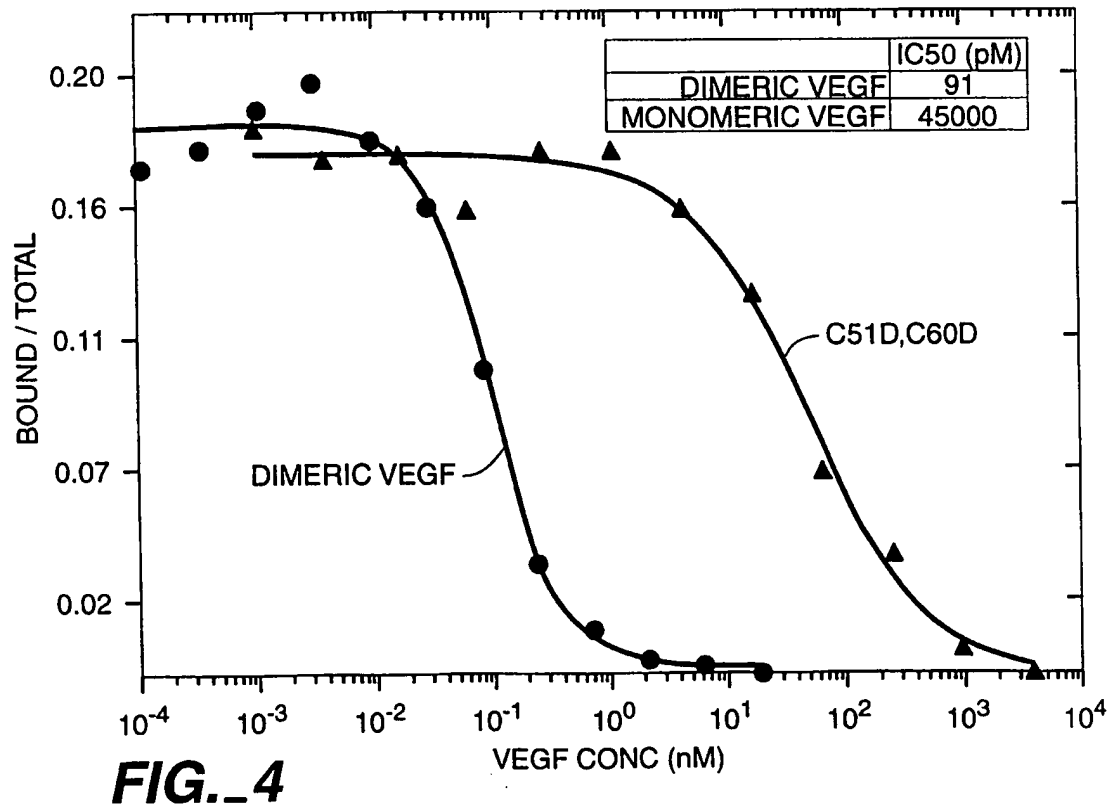

IC50 (pM)
DIMERIC VEGF 91
MONOMERIC VEGF 45000
0.20
0.16
0.11
0.07
0.02
BOUND / TOTAL
C51D,C60D
DIMERIC VEGF
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
10^4
VEGF CONC (nM)

FIG._4

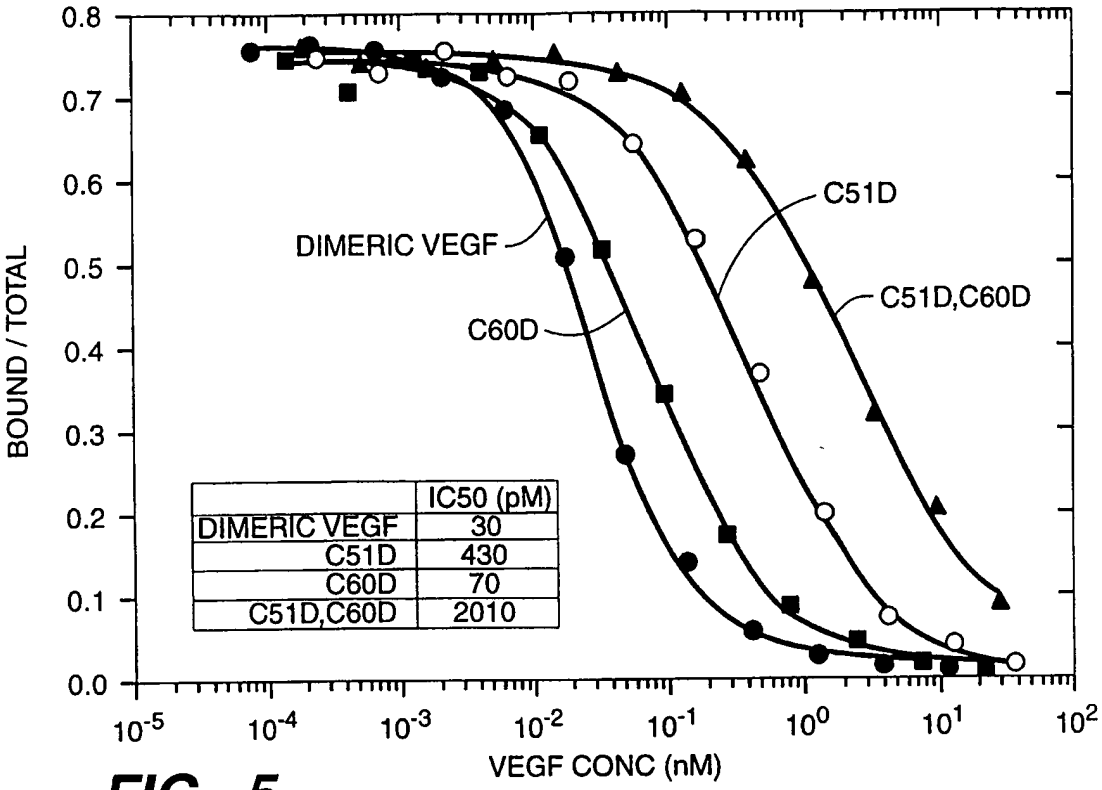

BOUND / TOTAL
VEGF CONC (nM)
DIMERIC VEGF
C51D
C51D,C60D
C60D
IC50 (pM)
DIMERIC VEGF 30
C51D 430
C60D 70
C51D,C60D 2010

FIG._5

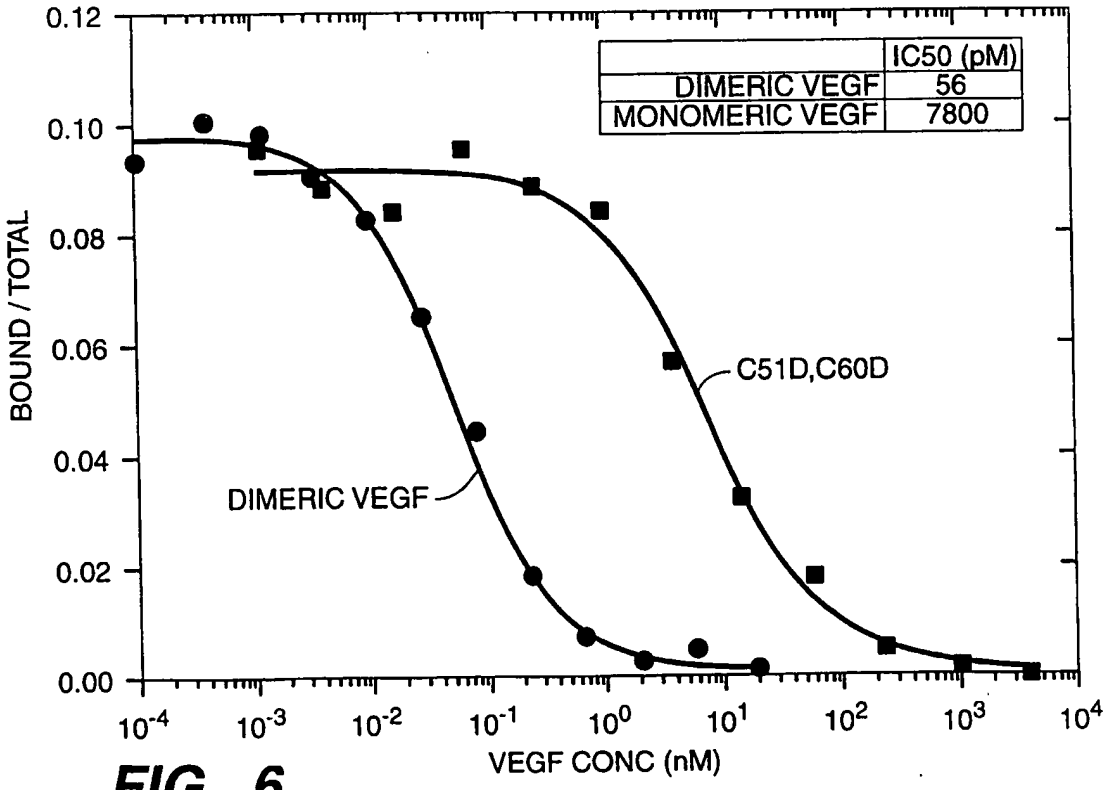

BOUND / TOTAL
VEGF CONC (nM)
IC50 (pM)
DIMERIC VEGF 56
MONOMERIC VEGF 7800
C51D,C60D
DIMERIC VEGF

FIG._6

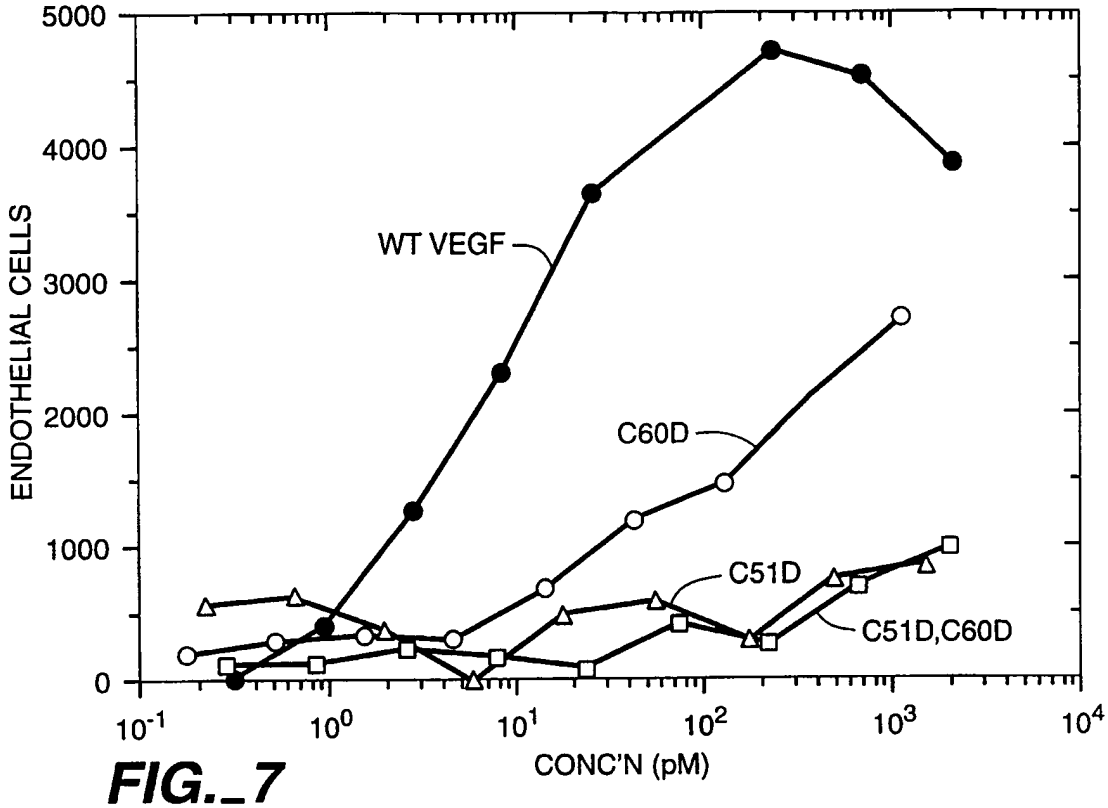

5000
4000
3000
2000
1000
0
ENDOTHELIAL CELLS
WT VEGF
C60D
C51D
C51D,C60D
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
CONC'N (pM)

FIG._7

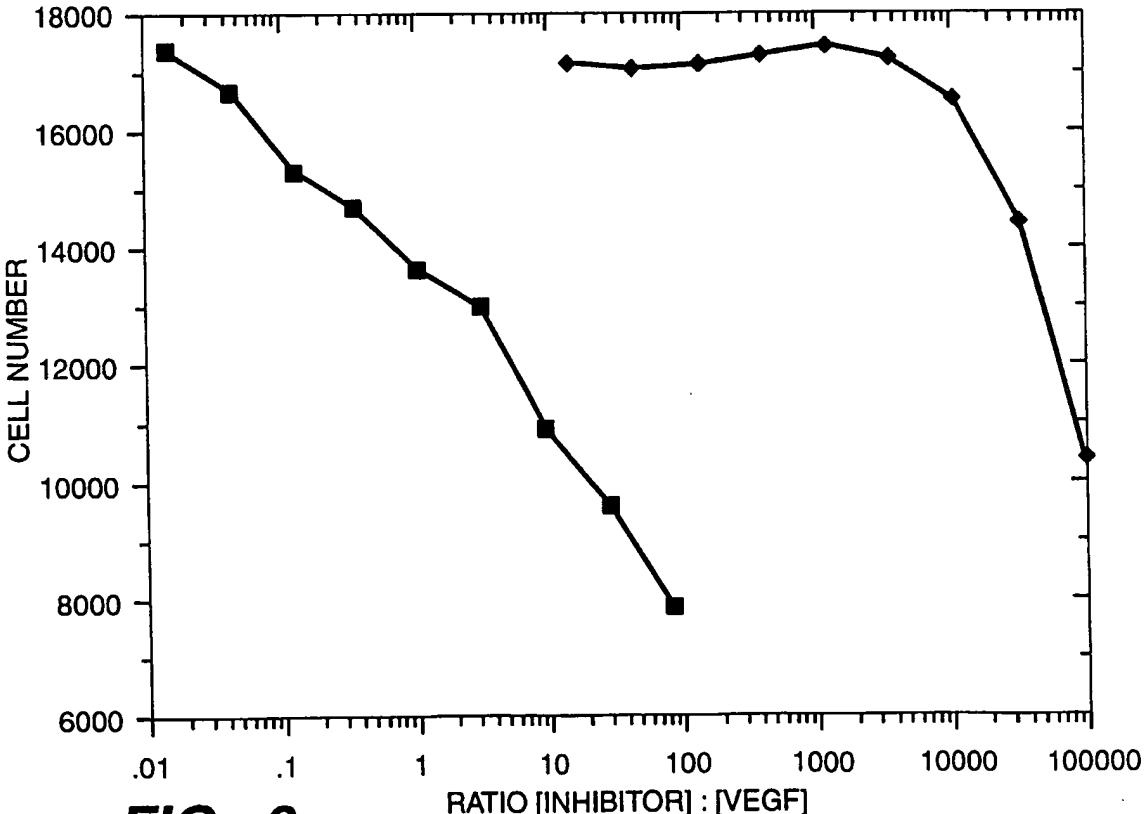

18000
16000
14000
12000
10000
8000
6000
CELL NUMBER
.01
.1
1
10
100
1000
10000
100000
RATIO [INHIBITOR] : [VEGF]

FIG._8

M&G No. 7780.823US01(3M Ref. 57885)

CHOLESTERIC LIQUID CRYSTAL ADDITIVES

Field of the Invention

The invention generally relates to cholesteric liquid crystal compositions for forming cholesteric liquid crystal films and optical bodies such as reflective polarizers, and particularly relates to additives for inclusion in cholesteric liquid crystal compositions.

Background of the Invention

Optical devices, such as polarizers and mirrors, are useful in a variety of applications including liquid crystal displays (LCD's). Liquid crystal displays fall broadly into two categories: backlit (e.g., transmissive) displays, where light is provided from behind the display panel, and frontlit (e.g., reflective) displays, where light is provided from the front of the display (e.g., ambient light). These two display modes can be combined to form transflective displays that can be backlit, for example, under dim light conditions or read under bright ambient light.

Conventional backlit LCDs typically use absorbing polarizers and can have less than 10% light transmission. Conventional reflective LCDs are also based on absorbing polarizers and typically have less than 25% reflectivity. The low transmission or reflectance of these displays reduces display brightness and can require high power consumption.

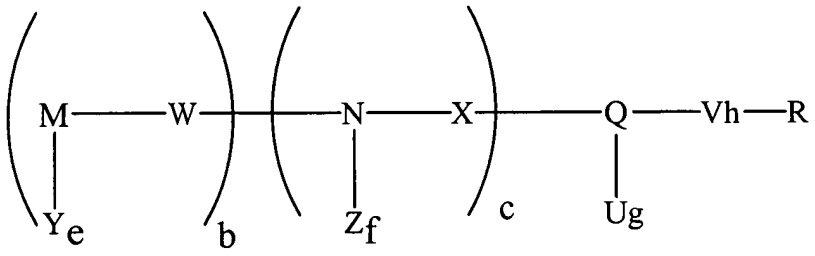
Reflective polarizers have been developed for use in these displays as well as other applications. Reflective polarizers preferentially reflect light having one polarization and transmit light of the opposite or orthogonal polarization. The reflected light has the ability to be recycled, making it possible to have a higher percentage of the light converted to polarized light and consequently a higher transmission of light. It is preferred that reflective polarizers transmit and reflect light without absorbing relatively large amounts of the light. Preferably, the reflective polarizer has no more than 10%

absorption for the transmission polarization over the desired range of wavelengths. Most LCD's operate over a broad range of wavelengths and, as a consequence, the reflective polarizer must typically operate over that broad wavelength range, as well.

The invention provides compositions and methods of making optical bodies for use in such applications.

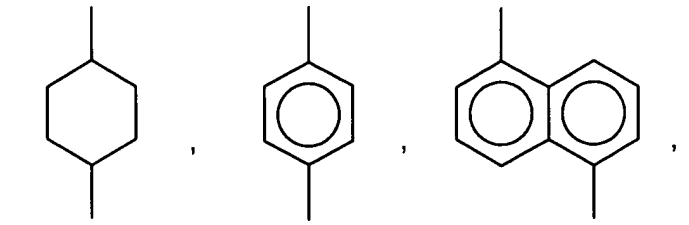
Summary of the Invention

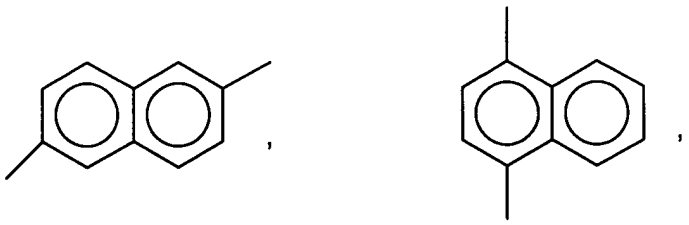
One embodiment of the invention provides a cholesteric liquid crystal composition that includes at least one cholesteric liquid crystal precursor, and at least one non-liquid crystalline additive of formula I

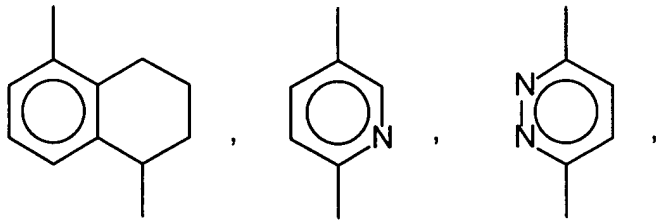


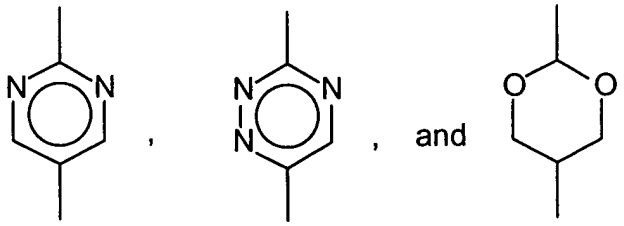
(I)

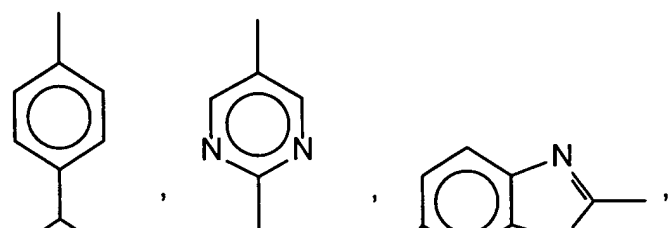
where M, N, and Q are each independently:



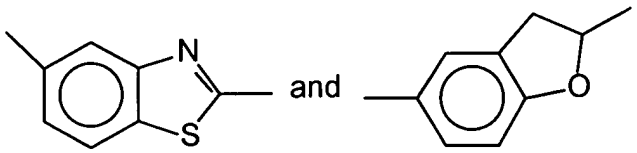


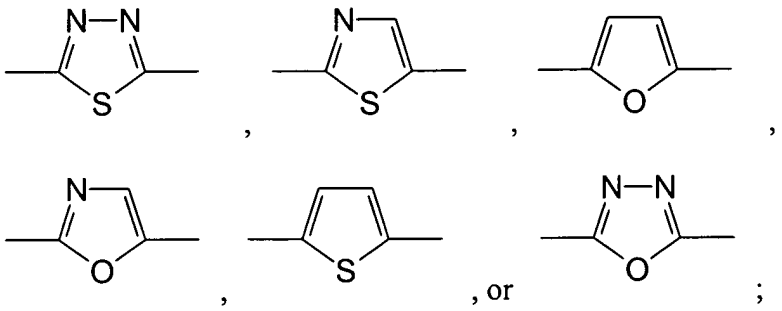






and





, , , , , , or ;

b and c are each independently zero or 1;

W, X, and V are each non-directionally and independently a covalent bond, -C(=O)-O-, -C(=O)-S-, -C(=O)-Se-, -C(=O)-Te-, $-(CH_2)_k-$ where k is 1 to 8, $-(CH=CH)_k-$where k is 1 to 8, $-(C\equiv C)_k-$ where k is 1 to 8, -CH=N-, $-CH_2-O-$, -C(=O)-, or -O- or a combination thereof;

Y, Z, and U are each independently -H, -Cl, -F, -Br, -I, -OH, $-O(CH_2)_jCH_3$ where j is 0 to 8, $-CH_3$, $-CF_3$, $-OCF_3$, -CN, $-NO_2$, aryl, arylalkoxy, carboxylic acid, thioether, or an amide;

e, f, and g are each independently zero, 1, 2, 3, or 4;

h is 1, 2, or 3; and

R is -H, -OH, -CN, C_1-C_8 alkyl, C_1-C_8 alkoxy, an aryl group, an arylalkoxy group, a carboxylic acid group, a halogen, a thioether, or an amide.

Another embodiment of the invention provides cholesteric liquid crystal films formed from a cholesteric liquid crystal composition of the invention. Optical bodies that include a reflective polarizer including a cholesteric liquid crystal formed from a cholesteric liquid crystal composition of the invention are also provided.

The above summary of the invention is not intended to describe each disclosed embodiment or every implementation of the invention. The Figures and the detailed description which follow more particularly exemplify these embodiments.

Brief Description of the Drawings

The invention may be more fully understood in consideration of the following detailed description of various embodiments of the invention in connection with the accompanying drawings in which:

Figure 1 is a schematic illustration of one embodiment of a liquid crystal display in accordance with the invention; and

Figure 2 is a schematic illustration of another embodiment of a liquid crystal display in accordance with the invention;

Figure 3 is a schematic illustration of another embodiment of a liquid crystal display in accordance with the invention.

While the invention is amenable to various modifications and alternative forms, specifics thereof have been shown by way of example in the drawings and will be described in detail. It should be understood, however, that the intention is not to limit the invention to the particular embodiments described herein. On the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention.

Detailed Description of the Preferred Embodiment

Cholesteric Liquid Crystal Compositions

Generally, a cholesteric liquid crystal composition in accordance with the invention includes at least one cholesteric liquid crystal precursor and at least one non-liquid crystalline additive. A cholesteric liquid crystal composition can also contain at least one solvent, and may contain compounds that function as initiators, terminators, curing agents, crosslinkers, antiozonants, antioxidants, plasticizers, stabilizers, and ultraviolet, infrared, or visible light-absorbing dyes and pigments. Cholesteric liquid crystal compositions of the invention can be used to form cholesteric liquid crystal layers.

Cholesteric liquid crystal precursors generally include chiral molecules or moieties or a mixture of chiral and achiral molecules and can be monomers, polymers or oligomers. Such precursors generally include at least one molecular unit that is

chiral in nature (i.e., does not possess a plane of symmetry) and at least one molecular unit that is mesogenic in nature (i.e, exhibits a liquid crystal phase). Cholesteric liquid crystal precursors can also be referred to as chiral nematic liquid crystal compounds. Thus cholesteric liquid crystal compositions include precursors having a cholesteric liquid crystal phase in which the molecular and optical director (i.e., the unit vector in the direction of average local molecular alignment) of the liquid crystal rotates in a helical fashion along the dimension (the helical axis) perpendicular to the director.

The pitch of the cholesteric liquid crystal layer is the distance (in a direction perpendicular to the director) that it takes for the director or mesogen to rotate through 360°. This distance is generally 250 to 600 nm or more. The pitch of a cholesteric liquid crystal precursor can typically be altered by mixing or otherwise combining (e.g., by copolymerization) in various proportions, at least one chiral compound (e.g., a cholesteric liquid crystal compound) with another typically nematic liquid crystal compound. In such a case, the pitch depends on the relative ratios, by molarity or weight, of the chiral compound in the cholesteric liquid crystal composition. The pitch is generally selected to be on the order of the wavelength of light of interest. The helical twist of the director results in a spatially periodic variation in the dielectric tensor, which in turn gives rise to the wavelength selective reflection of light. For example, the pitch can be selected such that the selective reflection occurs in the visible, ultraviolet, or infrared wavelengths of light.

Cholesteric liquid crystal precursors, including cholesteric liquid crystal polymers, are generally known and any cholesteric liquid crystal precursor known to those of skill in the art can be used in compositions of the invention. Examples of suitable cholesteric liquid crystal precursors are described in U.S. Patent Nos. 4,293,435, 5,332,522, 5,886,242, 5,847,068, 5,780,629, and 5,744,057, all of which are incorporated herein by reference. However, other cholesteric liquid crystal precursors not disclosed therein can also be utilized in compositions of the invention.

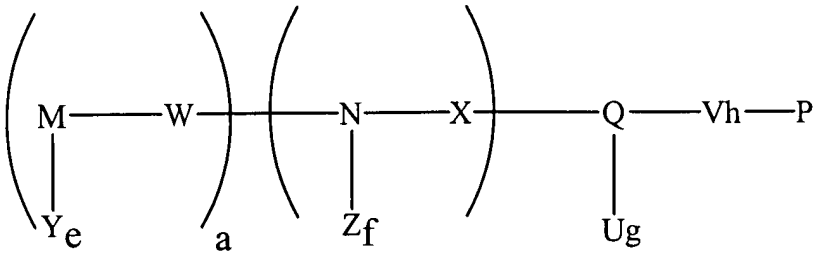
Generally, a cholesteric liquid crystal precursor is selected, at least in part, based on the particular application or optical body that it is ultimately to be used in. Examples of characteristics that can be considered in the choice of cholesteric liquid crystal

precursors include but are not limited to: refractive indices, pitch, processability, clarity, color, low absorption in the wavelength of interest, compatibility with other components (e.g., a nematic liquid crystal compound), ease of manufacture, availability of the cholesteric liquid crystal precursor or monomers to form the cholesteric liquid crystal polymer, rheology, method and requirements of curing, ease of solvent removal, physical and chemical properties (e.g., flexibility, tensile strength, solvent resistance, scratch resistance, and phase transition temperature), and ease of purification.

Cholesteric liquid crystal precursors that are cholesteric liquid crystal polymers are typically formed using chiral molecules or a mixture of chiral and achiral molecules (including monomers) that include a mesogenic group. Mesogenic groups are generally a rigid rodlike or disclike molecule or portion of a molecule that are components of liquid crystals. Examples of mesogenic groups include, but are not limited to, para-substituted cyclic groups, such as para-substituted benzene rings. These mesogenic groups are optionally bonded to a polymer backbone through a spacer. The spacer can contain functional groups having, for example, benzene, pyridine, pyrimidine, alkyne, ester, alkylene, alkane, ether, thioether, thioester, and amide functionalities.

Suitable cholesteric liquid crystal polymers include, but are not limited to, polymers having a chiral or achiral polyester, polycarbonate, polyamide, polyacrylate, polymethacrylate, polysiloxane, or polyesterimide backbone that include mesogenic groups optionally separated by rigid or flexible comonomers. Examples of other suitable cholesteric liquid crystal polymers have a polymer backbone (for example, a polyacrylate, polymethacrylate, polysiloxane, polyolefin, or polymalonate backbone) with chiral and achiral mesogenic side-chain groups. The side-chain groups can be optionally separated from the backbone by a spacer, such as an alkylene or alkylene oxide spacer, to provide flexibility.

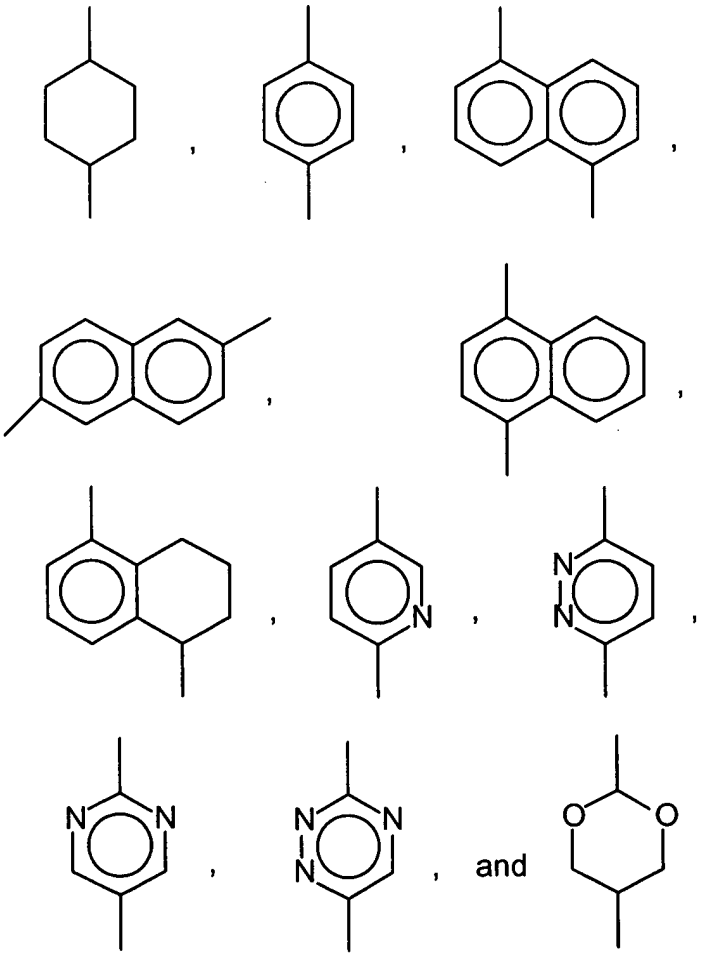
In one embodiment of the invention, a cholesteric liquid crystal precursor in accordance with the invention includes compounds of formula II



(II)

where a is 1, 2, or 3;

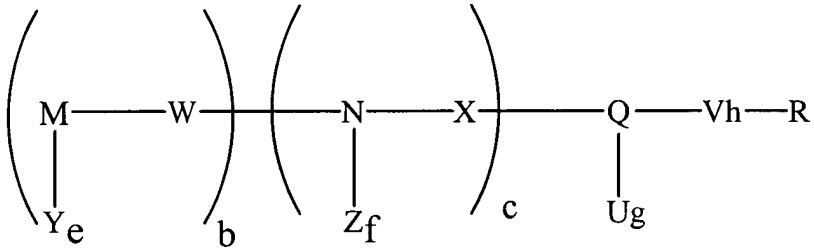
where M, N, and Q are each independently:



P is an acrylate, methacrylate, acrylamide, acrylonitrile, methacrylonitrile, amide, ester, urethane, ether, imide, or siloxane.

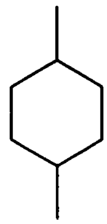
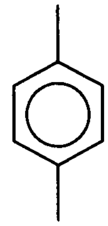
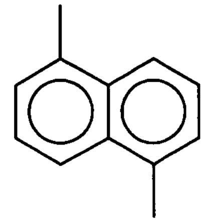
An example of a compound of formula II that may be utilized as a cholesteric liquid crystal precursor in a cholesteric liquid crystal composition of the invention includes, but is not limited to, cyanobiphenyl benzoate ethyl acrylate.

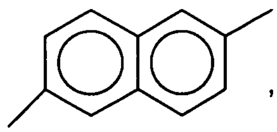
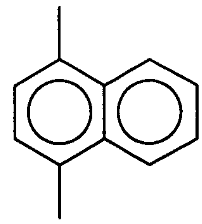
Cholesteric liquid crystal compositions of the invention also comprise at least one non-liquid crystalline additive of formula I

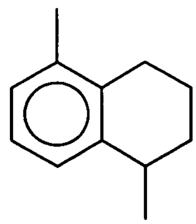
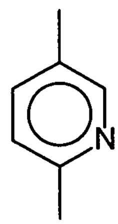
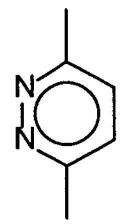


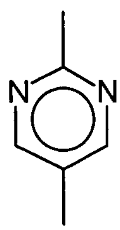
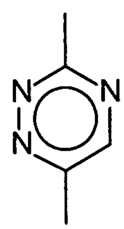
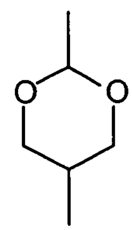
(I)

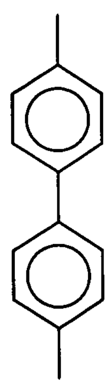
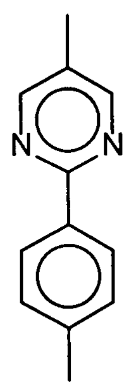
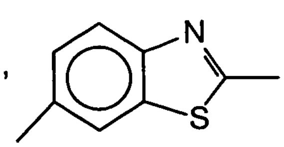
where M, N, and Q are each independently:

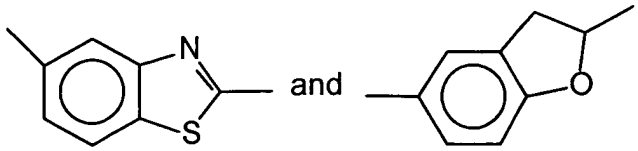
 ,  ,  ,

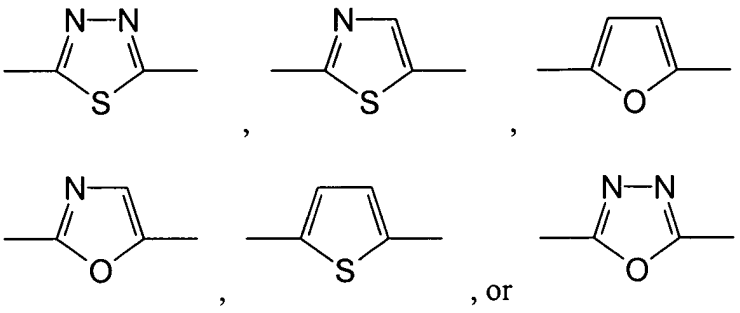
 ,  ,

 ,  ,  ,

 ,  , and 

 ,  ,  ,





, , , , , or ;

b and c are each independently zero or 1;

W, X, and V are each non-directionally and independently a covalent bond, -C(=O)-O-, -C(=O)-S-, -C(=O)-Se-, -C(=O)-Te-, $-(CH_2)_k-$ where k is 1 to 8, $-(CH=CH)_k-$where k is 1 to 8, $-(C\equiv C)_k-$ where k is 1 to 8, -CH=N-, $-CH_2-O-$, -C(=O)-, or -O- or a combination thereof;

Y, Z, and U are each independently -H, -Cl, -F, -Br, -I, -OH, $-O(CH_2)_jCH_3$ where j is 0 to 8, $-CH_3$, $-CF_3$, $-OCF_3$, -CN, $-NO_2$, aryl, arylalkoxy, carboxylic acid, thioether, or an amide;

e, f, and g are each independently zero, 1, 2, 3, or 4;

h is 1, 2, or 3; and

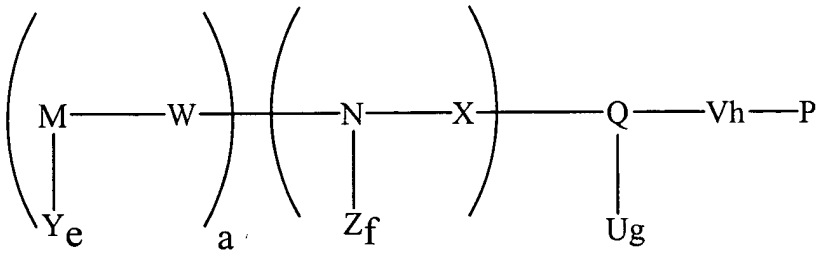
R is -H, -OH, -CN, C_1-C_8 alkyl, C_1-C_8 alkoxy, an aryl group, an arylalkoxy group, a carboxylic acid group, a halogen, a thioether, or an amide.

Additives for use in cholesteric liquid crystal compositions of the invention are non-liquid crystalline compounds. As used herein, a compound that is non-liquid crystalline is one that when melted forms an isotropic phase instead of a liquid crystalline phase.

Examples of compounds of formula I that may be utilized as a non-liquid crystalline additive in cholesteric liquid crystal compositions of the invention include, but are not limited to, 4'-cyano-4-biphenyl 4-methoxybenzoate, 4'-cyano-4-biphenyl 4-

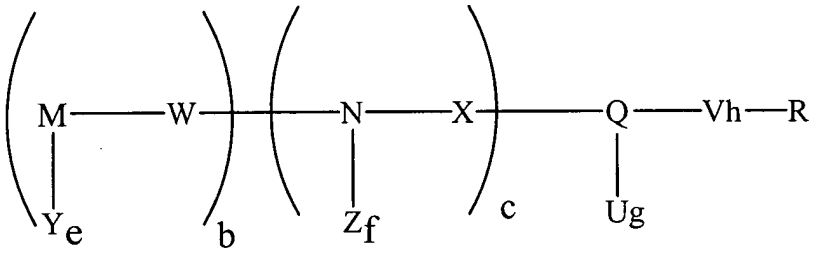
ethoxybenzoate, 4'-cyano-4-biphenyl 4-propoxybenzoate, 4'-cyano-4-biphenyl 4-butoxybenzoate, and 4'-cyano-4-biphenyl 4-heptoxybenzoate.

One embodiment of the invention includes a cholesteric liquid crystal composition that includes at least one cholesteric liquid crystal precursor of formula II



(II)

wherein M, N, P, Q, U, V, W, X, Y, Z, a, e, f, g, and h are as defined above, and at least one non-liquid crystalline additive of formula I



(I)

wherein M, N, Q, R, U, V, W, X, Y, Z, b, c, e, f, g, and h are as defined above, wherein at least Q is the same in both formula I and II.

In another embodiment, Q, U, V, g, and h are the same and b and c are 0. In yet another embodiment at least Q, U, V, X, N, Z, f, g, and h are the same and b is 0 and c is 1. In a further embodiment, at least Q, U, V, X, N, Z, W, M, Y, e, f, g, and h are the same and b, and c are 1.

Examples of compounds that can be utilized as cholesteric liquid crystal precursors of formula II and non-liquid crystalline additives of formula I in one of these embodiments include, but are not limited to, cyanobiphenyl benzoate ethyl acrylate as a cholesteric liquid crystal precursor of formula II, and 4'-cyano-4-biphenyl 4-methoxybenzoate, 4'-cyano-4-biphenyl 4-ethoxybenzoate, 4'-cyano-4-biphenyl 4-propoxybenzoate, 4'-cyano-4-biphenyl 4-butoxybenzoate, and 4'-cyano-4-biphenyl 4-heptoxybenzoate as non-liquid crystalline additives of formula I.

It should be understood by those of skill in the art having read this specification that cholesteric liquid crystal compositions of the invention can include more than one non-liquid crystalline additive of formula I, and can also include more than one cholesteric liquid crystal precursor of formula II.

In one embodiment of the invention, cholesteric liquid crystal compositions generally have from about 1 to 25% of one or more non-liquid crystalline additives of formula I by weight of the total composition. In another embodiment, the cholesteric liquid crystal composition has from about 10 to 20% of one or more non-liquid crystalline additives of formula I by weight of the total composition. In a further embodiment, the cholesteric liquid crystal composition has about 15% of one or more non-liquid crystalline additives of formula I by weight of the total composition.

The addition of non-liquid crystalline additives of formula I to cholesteric liquid crystal compositions of the invention is thought to aid in the alignment of the cholesteric liquid crystal precursors during the formation of a liquid crystal layer. Because non-liquid crystalline additives of formula I may serve to impart this characteristic to cholesteric liquid crystal compositions of the invention, they are often referred to as "compatible". One indication that the at least one cholesteric liquid crystal precursor is more uniformly aligned, i.e., that the non-liquid crystalline additive is compatible with the at least one cholesteric liquid crystal precursor can be seen by measuring a transmission of a dried film of the cholesteric liquid crystal composition that is not reduced as compared to a composition without the non-liquid crystalline additive. In another embodiment, the measured transmission is higher than that of a composition without the non-liquid crystalline additive.

The extinction of the cholesteric liquid crystal film that is formed from a cholesteric liquid crystal composition of the invention can be an indication of some of the properties of the cholesteric liquid crystal film that may ultimately be obtained from a cholesteric liquid crystal composition of the invention. In one embodiment, the extinction of a cholesteric liquid crystal film formed from a cholesteric liquid crystal composition of the invention is less than that of a cholesteric liquid crystal film formed from a cholesteric liquid crystal composition without the non-liquid crystalline additive.

In another embodiment, the extinction is not greater than about 15. In another embodiment, the extinction is at least 5% less than the composition without the additive.

One example of a method for carrying out this test of transmission includes the following procedure. The additive, which is to be determined compatible or not, is mixed with the cholesteric liquid crystal precursor in a suitable solvent. An exemplary composition for this mixture includes 17% cholesteric liquid crystal precursor, 3% additive, and 80% solvent. The solution, containing the compound and the cholesteric liquid crystal precursor, is coated on a clear plastic film. For example, a polyethylene terephthalate (PET) film commercially available from 3M (SCOTCHPAK™ type 718386, St. Paul, MN) or DuPont (Wilmington, DE). The coating is annealed at a temperature such that the cholesteric liquid crystal precursor is in its nematic phase and below the nematic to isotropic transition temperature of the liquid crystal polymer formed thereby. The solution should be coated in an amount to give a dried thickness of about 4 micrometers (μm).

The spectrum of the coated film is then measured by using a Lambda 900 spectrophotometer (Perkin Elmer, Shelton CT) with a Glan-Thomas polarizer and a Newport zero order achromatic 1/4 wave film in the optical path. The 1/4 wave axis is maintained at 45° to the polarizer axis. First the uncoated polyester film is placed facing the 1/4 wave plate in the light path and used as a blank and the instrument is zeroed. The sample is then placed in the light path after the 1/4 wave film. The polarizer is then oriented to give the maximum transmission (extinction) at 550nm and the spectra is recorded from 400nm to 500nm. The range of 400nm to 500nm is used since this covers the blue portion of the visible spectrum. Incompatible coatings produce haze which scatters the most in the blue portion of the spectrum. The spectra is averaged every 10nm in order to get a % pass transmission for the sample.

Cholesteric liquid crystal compositions in accordance with the invention can also include a solvent. In some instances, one or more of the cholesteric liquid crystal precursors or other components of the cholesteric liquid crystal composition can act as a solvent. The one or more solvents, or other compounds that function as a solvent are

generally substantially eliminated from the cholesteric liquid crystal composition during processing. They can be eliminated by, for example, drying the composition to evaporate the solvent or reacting a portion of the solvent (e.g., reacting a solvating liquid crystal monomer to form a liquid crystal polymer). Any solvents known to those of skill in the art as being able to solubilize the components of the composition can be used, specific examples include, but are not limited to, tetrahydrofuran, cyclohexanone, cyclopropanone, methyl ethyl ketone and 1,3-dioxolane. Combinations of solvents can also be utilized in compositions of the invention.

Cholesteric liquid crystal compositions in accordance with the invention can also include polymerization initiators that function to initiate polymerization or crosslinking of monomeric or other lower molecular weight compounds of the composition. Suitable polymerization initiators include compounds that can generate free radicals to initiate and propagate polymerization or crosslinking. Free radical initiators can be chosen based on a number of factors, including but not limited to, stability or half-life. Preferably, the free radical initiator does not generate any additional color in the cholesteric liquid crystal layer by absorption or otherwise.

Free radical initiators are typically either thermal free radical initiators or photoinitiators. Thermal free radical initiators, which generate free radicals upon thermal decomposition, include, for example, peroxides, persulfates, or azonitrile compounds. Photoinitiators can be activated by electromagnetic radiation or particle irradiation. Examples of suitable photoinitiators include, but are not limited to, onium salt photoinitiators, organometallic photoinitiators, cationic metal salt photoinitiators, photodecomposable organosilanes, latent sulphonic acids, phosphine oxides, cyclohexyl phenyl ketones, amine substituted acetophenones, and benzophenones. Generally, ultraviolet (UV) irradiation is used to activate the photoinitiator, although other light sources or irradiation (e-beam) can be used. Photoinitiators can be chosen based on the absorption of particular wavelength of light.

Cholesteric liquid crystal compositions in accordance with the invention may also additionally contain compounds that function as dispersing agents, terminators, curing agents, crosslinkers, antiozonants, antioxidants, plasticizers, stabilizers, viscosity

modifiers, such as thickeners and fillers, coalescing agents, which function to improve the intimacy of the contact between particles after deposition onto the substrate, and dyes and pigments for absorbing ultraviolet, infrared, or visible light.

Formation of Liquid Crystals

Any method known to those of skill in the art for forming a cholesteric liquid crystal composition of the invention into a cholesteric liquid crystal layer can be utilized. Furthermore, improvements in and changes to existing methods of forming cholesteric liquid crystal layers can also be utilized to form cholesteric liquid crystal layers from cholesteric liquid crystal compositions of the invention. The methods discussed below offer examples of methods and techniques that may be utilized to form cholesteric liquid crystal layers in accordance with the invention, and are not meant to limit the invention in any way.

One method of forming a cholesteric liquid crystal layer includes applying a cholesteric liquid crystal composition to a surface of a substrate. The cholesteric liquid crystal composition can be applied to the surface in a number of ways, including, but not limited to coating and spraying. Alternatively, the surface of the substrate can be oriented prior to being coated with the cholesteric liquid crystal layer. The substrate can be oriented using, for example, drawing techniques, rubbing with a rayon or other cloth, or lyotropic alignment (U.S. Patent No. 6,395,354). Photoalignment substrates are described in U.S. Patent Nos. 4,974,941, 5,032,009, 5,389,698, 5,602,661, 5,838,407, and 5,958,293.

After coating, the cholesteric liquid crystal composition is converted into a liquid crystal layer. Generally, this process includes drying the composition and annealing the dried composition, alternatively, these processes can occur somewhat simultaneously. The formation of a cholesteric liquid crystal layer can be accomplished by a variety of techniques including evaporation of solvent that is present; crosslinking the cholesteric liquid crystal composition; annealing or curing (e.g., polymerizing) the cholesteric liquid crystal composition using, for example, heat, radiation (e.g., actinic

radiation), light (e.g., ultraviolet, visible, or infrared light), an electron beam, or a combination of these or similar techniques.

One example of a process of forming a liquid crystal layer includes depositing the cholesteric liquid crystal composition on an oriented substrate. After deposition, the cholesteric liquid crystal composition is heated above the glass transition temperature of the composition to the liquid crystal phase. The composition is then cooled below the glass transition temperature and the composition remains in the liquid crystal phase.

Another process for forming a liquid crystal layer includes depositing the cholesteric liquid crystal composition on a substrate, and aligning the oligomeric liquid crystal by drying the composition to remove the solvent. Cholesteric liquid crystal compositions of the invention can be dried by any method known to those of skill in the art including those that restrict air flow. Examples of methods and/or apparatuses that restrict airflow can be found in U.S. Patent Nos. 5,581,905; 5,694,701; and 6,134,808, all of which are incorporated herein by reference.

Cholesteric liquid crystal compositions can be formed into a layer that substantially reflects light having one polarization (e.g., left or right circularly polarized light) and substantially transmits light having the other polarization (e.g., right or left circularly polarized light) over a particular bandwidth of light wavelengths. This characterization describes the reflection or transmission of light directed at normal incidence to the director of the cholesteric liquid crystal material. Light that is directed at other angles will typically be elliptically polarized by the cholesteric liquid crystal material and the Bragg reflection peak is typically blue-shifted from its on-axis wavelength. Cholesteric liquid crystal materials are generally characterized with respect to normally incident light, as done below, however, it will be recognized that the response of these materials can be determined for non-normally incident light using known techniques.

The cholesteric liquid crystal layer can be used alone or in combination with other cholesteric liquid crystal layers or other types of layers or devices to form an optical body, such as, for example, a reflective polarizer. Cholesteric liquid crystal polarizers are used in one type of reflective polarizer. The pitch of a cholesteric liquid

crystal polarizer is similar to the optical layer thickness of multilayer reflective polarizers. Pitch and optical layer thickness determine the center wavelength of the cholesteric liquid crystal polarizers and multilayer reflective polarizers, respectively. The rotating director of the cholesteric liquid crystal polarizer forms optical repeat units similar to the use of multiple layers having the same optical layer thickness in multilayer reflective polarizers.

The center wavelength, λ_0, and the spectral bandwidth, $\Delta\lambda$, of the light reflected by the cholesteric liquid crystal layer depend on the pitch, p, of the cholesteric liquid crystal. The center wavelength, λ_0, is approximated by:

$$\lambda_0 = 0.5(n_o + n_e)p$$

where n_o and n_e are the refractive indices of the cholesteric liquid crystal for light polarized parallel to the director of the liquid crystal (n_e) and for light polarized perpendicular to the director of the liquid crystal (n_o). The spectral bandwidth, $\Delta\lambda$, is approximated by:

$$\Delta\lambda = 2\lambda_0(n_e - n_o)/(n_e + n_o) = p(n_e - n_o).$$

Cholesteric liquid crystal polarizers have been previously formed by laminating or otherwise stacking two already-formed cholesteric liquid crystal layers, each disposed on an individual substrate, with different pitches (e.g., layers having different compositions, for example, different ratios by weight of chiral and nematic liquid crystal compounds or monomers). These two layers are heated to diffuse liquid crystal material between the layers. The diffusion of material between the two layers typically results in the pitch of the layers varying over a range between the individual pitches of the two layers.

This method, however, requires a substantial number of processing steps including separately forming each layer (e.g., individually drying or curing each layer), stacking (e.g., laminating) the layers, and then heating the layers to cause diffusion of liquid crystal material between the two layers. This also requires substantial processing time, particularly, in view of the time required for diffusion between the two previously formed liquid crystal layers which are typically polymeric in nature.

Display Examples

The cholesteric liquid crystal optical bodies can be used in a variety of optical displays and other applications, including transmissive (e.g., backlit), reflective, and transflective displays. For example, Figure 1 illustrates a schematic cross-sectional view of one illustrative backlit display system 400 including a display medium 402, a backlight 404, a cholesteric liquid crystal reflective polarizer 408, as described above, and an optional reflector 406. The display system optionally includes a wave plate as part of the cholesteric liquid crystal reflective polarizer or as a separate component to convert the circularly polarized light from the liquid crystal reflective polarizer to linearly polarized light. A viewer is located on the side of the display device 402 that is opposite from the backlight 404.

The display medium 402 displays information or images to the viewer by transmitting light that is emitted from the backlight 404. One example of a display medium 402 is a liquid crystal display (LCD) and polarizer that transmits only light of one polarization state.

The backlight 404 that supplies the light used to view the display system 400 includes, for example, a light source 416 and a light guide 418, although other backlighting systems can be used. Although the light guide 418 depicted in Figure 1 has a generally rectangular cross-section, backlights can use light guides with any suitable shape. For example, the light guide 418 can be wedge-shaped, channeled, a pseudo-wedge guide, etc. The primary consideration is that the light guide 418 be capable of receiving light from the light source 416 and emitting that light. As a result, the light 418 can include back reflectors (e.g., optional reflector 406), extraction mechanisms and other components to achieve the desired functions.

The reflective polarizer 408 is an optical film that includes at least one cholesteric liquid crystal optical body, as described above. The reflective polarizer 408 is provided to substantially transmit light of one polarization state exiting the light guide 418 and substantially reflect light of a different polarization state exiting the light guide 418.

Figure 2 is a schematic illustration of one type of reflective liquid crystal display 500. This reflective liquid crystal display 500 includes a display medium 508, a cholesteric liquid crystal reflective polarizing mirror 504, an absorptive backing 506, and an absorptive polarizer 502. The liquid crystal display 500 optionally includes a wave plate as part of the cholesteric liquid crystal reflective polarizer 504 or as a separate component to convert mixed polarization light from the liquid crystal device to an appropriately polarized light.

Liquid crystal display 500 functions first by the absorptive polarizer 502 polarizing light 510. The polarized light then travels through the display medium 508 where one of the light's circular polarization components reflects from the cholesteric liquid crystal reflective polarizing mirror 504 and passes back through the display medium 508 and absorptive polarizer 502. The other circular polarization component passes through the cholesteric liquid crystal reflective polarizer 504 and is absorbed by the backing 506. The reflective polarizer 504 of this reflective liquid crystal display 500 includes one cholesteric liquid crystal optical body, as described above. The specific choice of cholesteric liquid crystal optical body can depend on factors such as, for example, cost, size, thickness, materials, and wavelength range of interest.

Figure 3 is a schematic illustration of one type of transflective liquid crystal display 600. This transflective liquid crystal display 600 includes a phase retarding display medium 608, a partial mirror 603, a cholesteric liquid crystal reflective polarizing mirror 604, a backlight 606, and an absorptive polarizer 602. The display system optionally includes a wave plate as part of the cholesteric liquid crystal reflective polarizer 604 or as a separate component to convert mixed polarization light from the liquid crystal device to an appropriately polarized light. In the reflective mode, bright ambient light 610 is polarized by the absorptive polarizer 602, travels through the display medium 608, reflects off the partial mirror 603, and passes back through the display medium 608 and absorptive polarizer 602. In low ambient-light situations, the backlight 606 is activated and light is selectively passed through the cholesteric polarizer 604, matched to provide appropriately polarized light to the display. Light of the opposite handedness is back-reflected, recycled, and selectively

passed through the cholesteric polarizer 604 to effectively increase backlight brightness. The reflective polarizer of this reflective liquid crystal display 600 includes one cholesteric liquid crystal optical body, as described above. The specific choice of cholesteric liquid crystal optical body can depend on factors such as, for example, cost, size, thickness, materials, and wavelength range of interest.

The cholesteric liquid crystal optical body can be used with a variety of other components and films that enhance or provide other properties to a liquid crystal display. Such components and films include, for example, brightness enhancement films, retardation plates including quarter-wave plates and films, multilayer or continuous/disperse phase reflective polarizers, metallized back reflectors, prismatic back reflectors, diffusely reflecting back reflectors, multilayer dielectric back reflectors, and holographic back reflectors.

Working Examples

All chemicals for the following examples were obtained from Aldrich, Inc. of Milwaukee, WI unless indicated otherwise.

Example 1: Synthesis of Various Non-liquid Crystalline Additives

4'-cyano-4'biphenyl 4-methoxybenzoate was prepared by first combining 40 g (0.263 moles) of 4-methoxybenzoic acid, 53.2 g (0.525 moles) and 400 ml of 1,2-dimethoxyethane in a round bottomed flask fitted with a mechanical stirrer and a thermometer, under an atmosphere of nitrogen. The solution was cooled to -30° C, at which point 30.1 g (0.263 moles) of methane sulfonyl cholride was added. Stirring was continued, and the temperature was maintained at -30° C for about 1 hour. Next, 51.32 g (0.263 moles) of 4'-cyano-4-biphenol and 3.2 g (0.026 moles) of 4-dimethylaminopyridene were added and the mixture was heated to 50° C and maintained at that temperature with stirring for about 3 hours. The mixture was then cooled to room temperature and 1 liter of H_2O was added. A solid product precipitated, was collected by filtration, and was washed with water and air dried. The crude

material was then recrystallized from a minimal amount of tetrahydrofuran to obtain the desired material.

Other exemplary additives were made using the same procedure by replacing the 4-methoxybenzoic acid with the chemical indicated in Table 1 below.

Table 1

Exemplary Additive	Chemical
4'-cyano-4'biphenyl 4-ethoxybenzoate	4-ethyoxybenzoic acid
4'-cyano-4'biphenyl 4-propoxybenzoate	4-propoxybenzoic acid
4'-cyano-4'biphenyl 4-butoxybenzoate	4-butoxybenzoic acid
4'-cyano-4'biphenyl 4-heptoxybenzoate	4-heptoxybenzoic acid

Example 2: Effect of Various Additives

18.5 wt% cyano biphenyl benzoate ethyl acrylate, 1.18 wt% Paliocolor LC 756 (BASF, Charlotte, NC), 59.2 wt% tetrahydrofuran, 19.6 wt% cyclohexanone, 0.93 wt% carbon tetrabromide, and 0.59% Vazo 52 (Wilmington, DE) were combined and heated at about 60° C for about 14 hours.

After 14 hours, the various non-liquid crystalline additives were added to the initial solution. The additives were added in an amount 15% of the weight of the initial solids. The resulting solutions were mixed until clear, at a temperature of 60° C. The mixed solutions were then coated on to 100 micron thick PET film (Scotch Pak c , 3M). The coated film was baked at about 120° C for about 12 minutes. The spectrum of each coated film was then measured by using a Lambda 900 spectrophotometer (Perkin Elmer, Sheton CT) with a Glan-Thomas polarizer and Newport zero order achromatic 1/4 wave film in the optical path. The polarizer was oriented to give the minimum transmission (extinction) and the spectra was recorded. The minimum in the spectra was averaged over 120 nm of bandwidth and is given in Table 1 below.

Table 2

Additive	Extinction (% Transmission)	Pass Transmission
control (no additive)	17.1	99.3
4-cyano-4'-hydroxybiphenyl	11.0	99.1
4'-cyano-4'-biphenyl 4-methoxybenzoate	9.5	99.3
4'-cyano-4'-biphenyl 4-ethoxybenzoate	10.1	99.0
4'-cyano-4'-biphenyl 4-propoxybenzoate	8.1	99.1
4'-cyano-4'-biphenyl 4-butoxybenzoate	9.6	99.2
4'-cyano-4'-biphenyl 4-heptoxybenzoate	29.1	98.2
cyanobiphenyl benzoate	10.6	99.2

As can be seen from Table 1, while all of the additives that were utilized were compatible with the liquid crystal polymer (they all had pass transmission of >90%) the cholesteric liquid crystal films formed thereby had varying results for extinction. They all produced cholesteric liquid crystal layers with a lower extinction, versus the composition without any additive (control), except for the composition with cyanobiphenyl heptoxy benzoate.

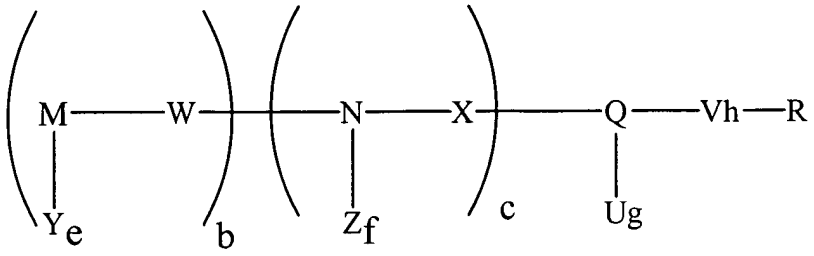
The above specification, examples and data provide a complete description of the manufacture and use of the composition of the invention. Since many embodiments of the invention can be made without departing from the spirit and scope of the invention, the invention resides in the claims hereinafter appended.

WE CLAIM:

1. A cholesteric liquid crystal composition comprising:

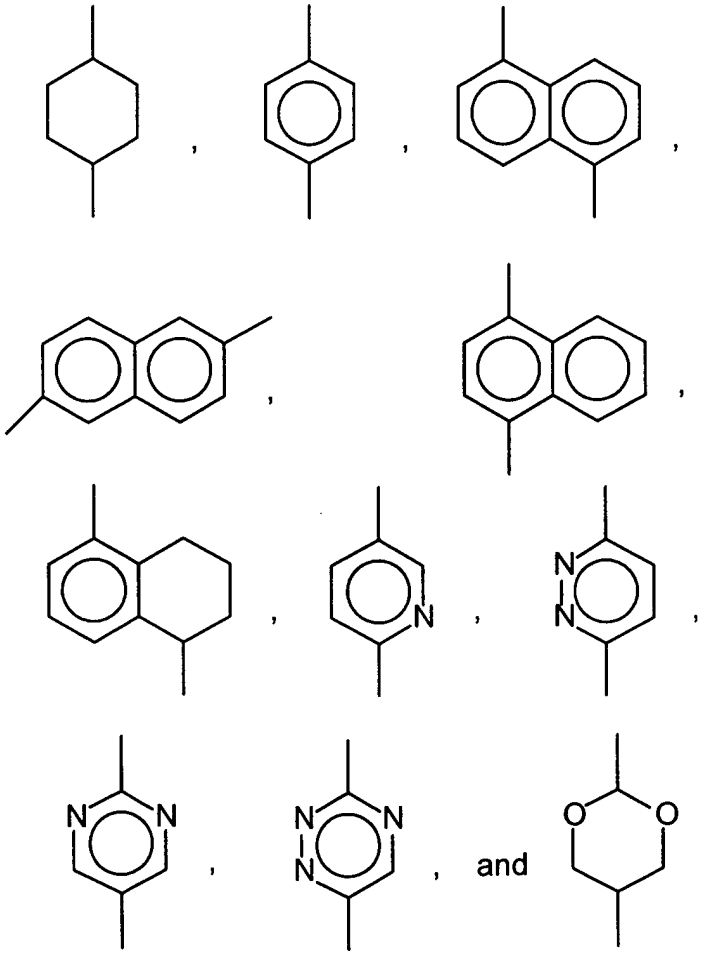
at least one cholesteric liquid crystal precursor; and

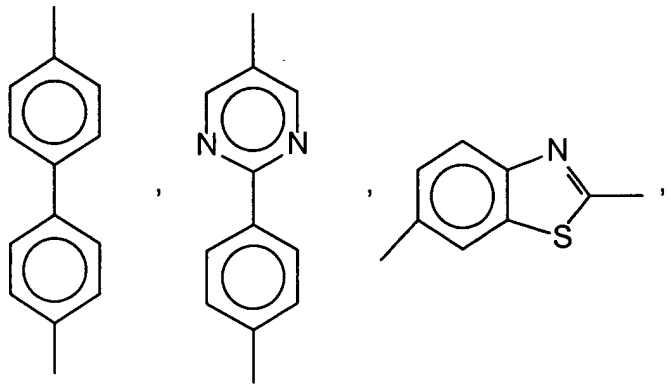
at least one non-liquid crystalline additive of formula I

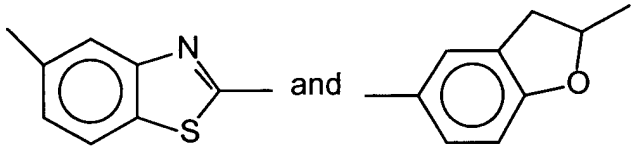


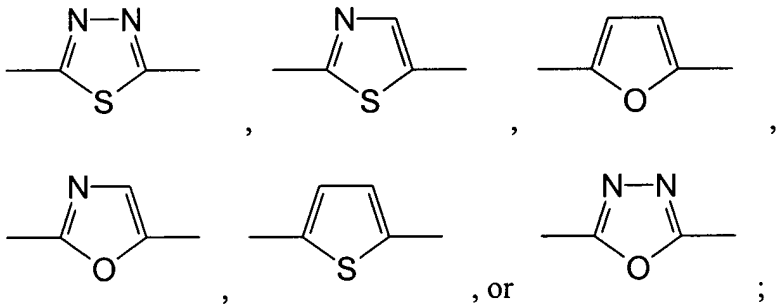
(I)

where M, N, and Q are each independently:









b and c are each independently zero or 1;

W, X, and V are each non-directionally and independently a covalent bond, -C(=O)-O-, -C(=O)-S-, -C(=O)-Se-, -C(=O)-Te-, $-(CH_2)_k-$ where k is 1 to 8, $-(CH{=}CH)_k-$where k is 1 to 8, $-(C{\equiv}C)_k-$ where k is 1 to 8, -CH=N-, $-CH_2-O-$, -C(=O)-, or -O- or a combination thereof;

Y, Z, and U are each independently -H, -Cl, -F, -Br, -I, -OH, $-O(CH_2)_jCH_3$ where j is 0 to 8, $-CH_3$, $-CF_3$, $-OCF_3$, -CN, $-NO_2$, aryl, arylalkoxy, carboxylic acid, thioether, or an amide;

e, f, and g are each independently zero, 1, 2, 3, or 4;

h is 1, 2, or 3; and

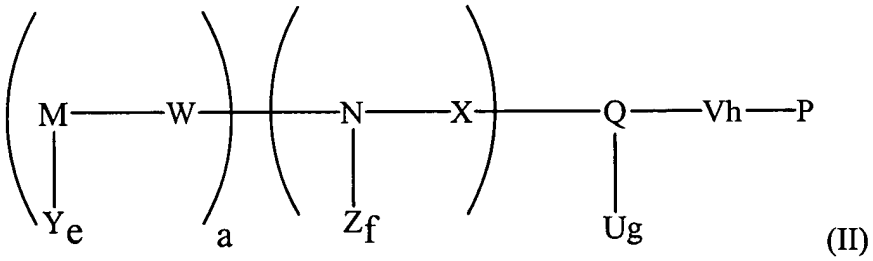
R is -H, -OH, -CN, C_1-C_8 alkyl, C_1-C_8 alkoxy, an aryl group, an arylalkoxy group, a carboxylic acid group, a halogen, a thioether, or an amide.

2. The composition according to claim 1, wherein the cholesteric liquid crystal composition has a transmission that is not greater than that of the cholesteric liquid crystal composition without the additive.

3. The composition according to claim 1, wherein a cholesteric liquid crystal film formed from the cholesteric liquid crystal composition has an extinction that is less than that of a film formed from the cholesteric liquid crystal composition without the additive.

4. The composition according to claim 1, wherein the film formed from the cholesteric liquid crystal composition has an extinction that is at least 5% less than the cholesteric liquid crystal composition without the additive.

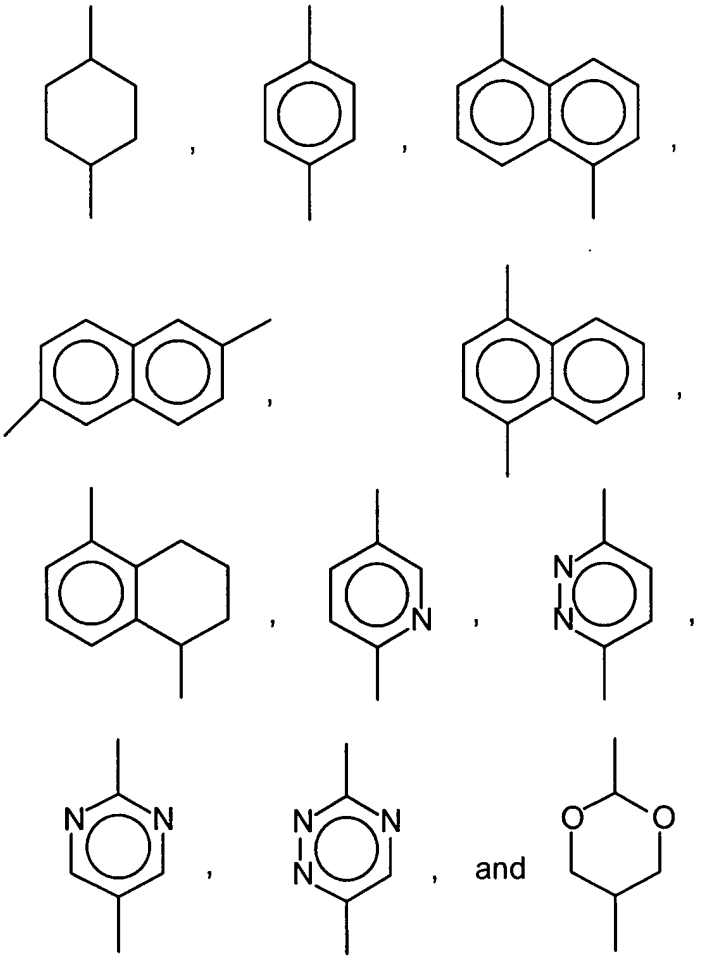
5. The composition according to claim 1, wherein the at least one cholesteric liquid crystal precursor comprises a compound of formula II

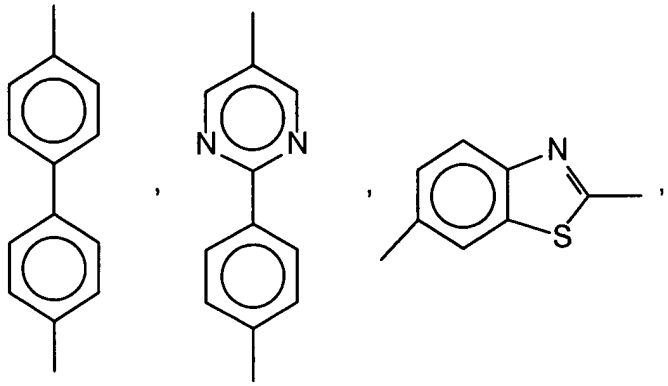


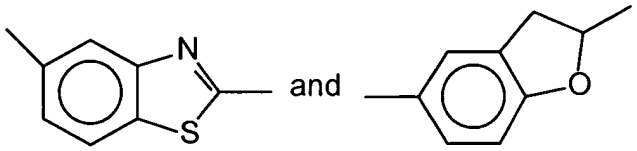
(II)

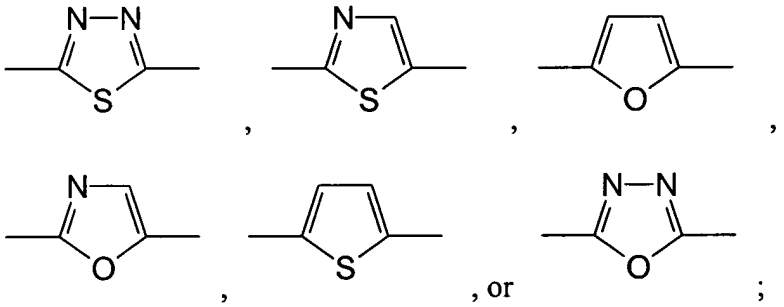
where a is 1, 2, or 3;

where M, N, and Q are each independently:


N
N
N
N
N
N
O
O
and







W, X, and V are each non-directionally and independently a covalent bond, -C(=O)-O-, -C(=O)-S-, -C(=O)-Se-, -C(=O)-Te-, $-(CH_2)_k-$ where k is 1 to 8, $-(CH=CH)_k-$ where k is 1 to 8, $-(C\equiv C)_k-$ where k is 1 to 8, -CH=N-, $-CH_2-O-$, -C(=O)-, -O-, or a combination thereof;

Y, Z, and U are each independently -H, -Cl, -F, -Br, -I, -OH, $-O(CH_2)_jCH_3$ where j is 0 to 8, $-CH_3$, $-CF_3$, $-OCF_3$, -CN, $-NO_2$, aryl, arylalkoxy, carboxylic acid, thioether, or an amide;

e, f, and g are each independently zero, 1, 2, 3, or 4; and

h is 1, 2, or 3; and

P is an acrylate, methacrylate, acrylamide, acrylonitrile, methacrylonitrile, amide, ester, urethane, ether, imide, or siloxane.

6. The composition according to claim 5, wherein at least Q is the same in both formula I, and II.

7. The composition according to claim 5, wherein Q, U, V, g, and h are the same in both formula I, and II; and b and c are both 0.

8. The composition according to claim 5, wherein at least Q, U, V, X, N, Z, f, g, and h are the same in both formula I and II; and b is 0 and c is 1 in formula I.

9. The composition according to claim 5, wherein at least Q, U, V, X, N, Z, W, M, Y, e, f, g, and h are the same in both formula I and II and b, and c are 1 in formula I.

10. The composition according to claim 5, wherein the cholesteric liquid crystal precursor is cyanobiphenyl benzoate ethyl acrylate and the additive is 4'-cyano-4-biphenyl 4-methoxybenzoate, 4'-cyano-4-biphenyl 4-ethoxybenzoate, 4'-cyano-4-biphenyl 4-propoxybenzoate, 4'-cyano-4-biphenyl 4-butoxybenzoate, 4'-cyano-4-biphenyl 4-heptoxybenzoate, or a combination thereof.

11. The composition according to claim 5, wherein the amount of the additive of formula I is about 1 to 25% by weight of the total composition.

12. The composition according to claim 5, wherein the amount of the additive of formula I is about 10 to 20% by weight of the total composition.

13. The composition according to claim 5, wherein the amount of the additive of formula I is about 15% by weight of the total composition.

14. The cholesteric liquid crystal composition according to claim 1 further comprising a solvent.

15. The cholesteric liquid crystal composition according to claim 1 further comprising at least one compound that functions as an initiator, a terminator, a curing agent, a crosslinker, an antiozonant, an antioxidant, a plasticizer, a stabilizer, a light absorbing dye, or a pigment.

16. A cholesteric liquid crystal layer formed from a cholesteric liquid crystal composition in accordance with claim 1.

17. An optical body comprising a cholesteric liquid crystal layer in accordance with claim 16.

18. An optical display comprising:

a display medium; and

a reflective polarizer comprising a cholesteric liquid crystal composition according to claim 1.

19. An optical display comprising:

a display medium; and

a reflective polarizer comprising a cholesteric liquid crystal composition according to claim 16.

20. The optical display according to claim 19, wherein at least Q is the same in both formula I, and II.

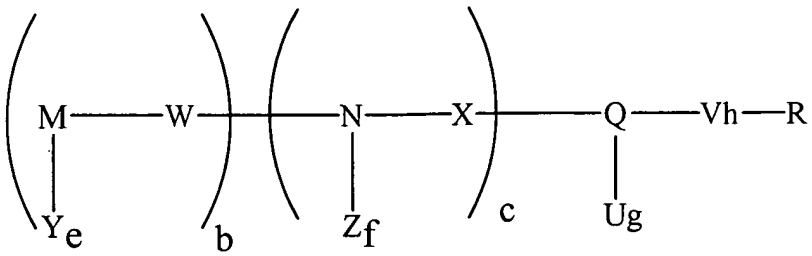
21. The optical display according to claim 19, wherein Q, U, V, g, and h are the same in both formula I, and II; and b and c are both 0.

22 The optical display according to claim 19, wherein at least Q, U, V, X, N, Z, f, g, and h are the same in both formula I and II; and b is 0 and c is 1 in formula I.

23. The optical display according to claim 19, wherein at least Q, U, V, X, N, Z, W, M, Y, e, f, g, and h are the same in both formula I and II and b, and c are 1 in formula I.

Abstract

The invention provides a cholesteric liquid crystal composition that includes at least one cholesteric liquid crystal precursor, and at least one non-liquid crystalline additive that is a non-liquid crystalline compound of formula I



The invention also provides cholesteric liquid crystal films and optical bodies formed from cholesteric liquid crystal compositions of the invention. The invention further provides an optical display that includes a display medium, and a reflective polarizer including a cholesteric liquid crystal composition in accordance with the invention.

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